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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 48/00, C07K 14/62

A2

(11) International Publication Number: WO 95/27512

(43) International Publication Date: 19 October 1995 (19.10.95)

(21) International Application Number: PCT/US95/04397

(22) International Filing Date: 10 April 1995 (10.04.95)

(30) Priority Data:
08/225,840 11 April 1994 (11.04.94) US

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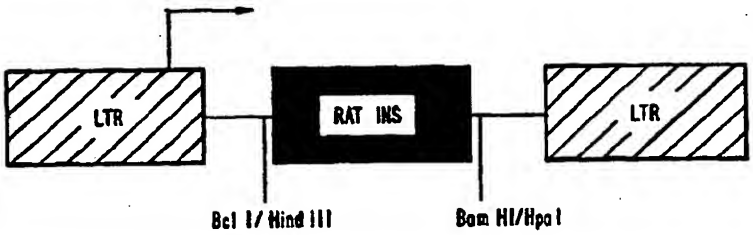
(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

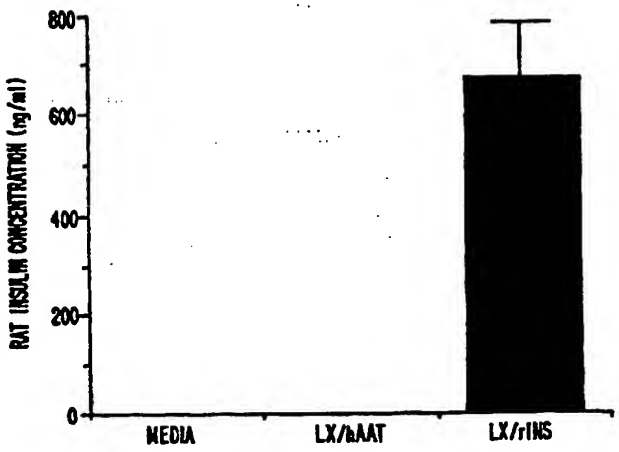
Published
Without international search report and to be republished upon receipt of that report.

(54) Title: COMPOSITIONS AND METHODS FOR GENE THERAPY TO TREAT DISEASE

(57) Abstract

Method of gene therapy for the treatment of a variety of genetic defects or diseases comprising the steps of either introducing transformed cells into an organism such as a human or the *in vivo* transformation of cells. The transformed cells contain a nucleic acid vector which will produce the protein necessary to correct the deficiency or disease. Examples of diseases which can be treated this way include diabetes mellitus, Parkinson's disease, cardiovascular disease, hypercholesterolemia, hypertension, anemia, thrombosis, growth disorders, metabolic disorders such as PKU and serum protein disorders, such as hemophilia A.





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DESCRIPTION

Compositions and Methods for Gene Therapy to Treat Disease

Background of the Invention

The present invention generally relates to the use of gene therapy in the treatment of various diseases. More specifically, it relates to introducing transformed cells or nucleic acid vectors into animals or *in vivo* transformation of cells to treat diabetes mellitus, Parkinson's disease, cardiovascular disease, hypercholesterolemia, hypertension, anemia, thrombosis, growth disorders, metabolic disorders such as phenylketonuria ("PKU") and serum protein disorders, such as hemophilia A.

Somatic gene therapy is a method of transferring recombinant genes into somatic cells to replace genetically defective functions or to otherwise alter the course of a pathological disease process.

There is a considerable need for the development of improved therapies for a number of diseases. Type 1 or insulin dependent diabetes mellitus ("IDDM") is caused by a lack of insulin and results from the autoimmune destruction of the insulin producing β-cells of the pancreas (Huang, S-W, and Maclaren, N.K., 1976, Science 192: 64). Insulin is a key signalling molecule whose concentration controls the body's stores of energy reserves in the form of glycogen, protein and fat (Cahill, G.F., 1971, Diabetes 20:785). In IDDM, the lack of insulin results in unnecessary mobilization of energy reserves and poor utilization of available fuels, which leads to wasting, high blood glucose levels, and production of ketone bodies (Cahill, G.F., 1971, Diabetes 20:785). Acute death from uncontrolled IDDM is caused by ketoacidosis (Joslin, E.P., et al., 1925, Med. Clin. N. Amer. 8:1873), characterized by high levels of ketones (greater than 5 mmol/L) and low blood pH (plasma

bicarbonate levels less than 9 mEq/L) (Vignati, L., et al., Chapter 26. Coma and Diabetes. In: Marble, A., Krall, L.P., Bradley, R.F., Christlieb, A.R., and Soeldner, J.S. (Eds.): Joslin's Diabetes Mellitus. Philadelphia, Lea & Febiger 1985, pp. 526). The present treatment for IDDM includes daily monitoring of blood glucose levels, injections of insulin, and restricted diet and exercise regimens (Bergman, M. (Ed.): Principles of Diabetes Management. Medical Publishing Company 1987, pp. 42-85). Even with vigorous compliance, diabetic patients can expect severe health complications, and their life expectancy is only two thirds of that of non-diabetic individuals (Olefsky, J.M., and Sherwin, R.S. (Eds.): Diabetes Mellitus: Management and Complications. Churchill Livingstone, 1985). A new treatment modality for diabetes by gene therapy would be beneficial.

Idiotypic Parkinson's Disease is caused by the degeneration of dopaminergic cells of the nigrostriatal pathway in the human brain. These cells are involved in the regulation of muscle control and upon their degeneration, the classical Parkinsonian symptoms of tremor, rigidity and akinesia are unmasked. The etiology of the neuronal degeneration in Parkinson's Disease is unknown.

The most commonly employed therapy involves a peripheral administration of L-Dopa, the direct biochemical precursor to dopamine. Unlike dopamine, L-Dopa can cross the blood brain barrier to enter the brain from the peripheral circulation. Once inside the brain, the L-Dopa is taken up in the neurons where it is decarboxylated to form dopamine. However, dramatic fluctuations in blood levels of L-Dopa associated with oral dosing schedules can occur. This contributes to the occurrence of a number of untoward side effects. Thus an improved method of treatment of Parkinson's disease would be beneficial.

Hypercholesterolemia contributes to a number of diseases, including coronary vascular disease, cerebral vascular disease, peripheral vascular disease and incapacitation or death by myocardial infarction and stroke. Identification of the molecular defect which causes familial hypercholesterolemia ("FH") provided insight into lipid metabolism and aided in the development of new treatments for hyperlipidemia and atherosclerosis. Low density lipoprotein ("LDL"), a metabolic product of triglyceride-rich very low density lipoprotein ("VLDL"), is cleared from the plasma primarily by receptor-mediated endocytosis into hepatocytes. Patients with FH have mutations in the LDL receptor gene that block either the synthesis or transport of the LDL receptor to the cell surface, inhibit LDL binding to the receptor, or prevent internalization of the LDL-receptor complex. FH heterozygotes, who comprise one out of every 500 individuals in the US population, exhibit two-fold elevations in plasma cholesterol levels, and often begin to develop tendon xanthomas and coronary atherosclerosis around 20 years of age (Epstein, F.H., et al., 1959. *Am. J. Med.* 26:39-531; Goldstein, J. L., M. S. Brown. 1974. *J. Biol. Chem.* 249:5153-62). FH homozygotes, who comprise one out of every one million individuals in the US population, exhibit four-fold elevations in plasma cholesterol levels, often develop cutaneous xanthomas before 4 years of age, display well-developed coronary heart disease in childhood, and frequently die before age 20 (Khachadurian, A. K. 1964. *Am. J. Med.* 37:402-7; Khachadurian, A. K., S. M. Uthman. 1973. *Nutr. Metab.* 15:132-40).

Current treatments for FH heterozygotes have focused on up-regulating expression of the normal LDL receptor gene (Goldstein, J. L., et al., 1976. *Cell* 9:195-203). Drugs that block bile acid recycling and cholesterol biosynthesis are frequently used to decrease intracellular cholesterol levels. These treatments result in

up-regulation of LDL receptor gene expression, leading to a decrease in plasma cholesterol levels (Hashim, S. A., T. B. Van Itallie. 1965. *JAMA* 192:289-293; Endo, A., et al., 1976. *FEBS Lett.* 72:323-326; Kovanen, P. T., et al., 1981. *Proc. Natl. Acad. Sci. USA* 78:1194-1198). This therapy is much less effective in FH homozygotes, since neither copy of the LDL receptor gene produces a fully functional receptor. These patients have been treated by plasma exchange for removal of LDL (Thompson, G. R., et al., 1975. *Lancet* 1:1208-11) and portacaval anastomosis (Starzl T. E., et al., 1973. *Lancet* 2:940-944) with varying degrees of success. Therefore an alternative, improved method of treating hypercholesterolemia is needed.

Hypertension is a heterogeneous disorder, in which a variety of genetic and environmental factors interact and result in an increase in blood pressure (Hachinski, V.C., 1991, AJH 4:118S-120S.) In the US, 38%, 46% and 16% of the white, black and Hispanic males, respectively, are hypertensive. For women, the percentages are 28%, 47%, and 12%, respectively, for the same ethnic groups (Sorel, J.E., et al., 1991, Am. J. Epidemiol. 134:370-378). The cause(s) of primary arterial hypertension in humans and of spontaneously appearing hypertension in animals have not been identified to date (Oparil, S., Chen, Y.-F., Natyilan, A.J., and Wyss, J.M., 1991, Pathogenesis of Hypertension in The Heart and Cardiovascular System, 2nd edition (Fozzard, H.A., Haber, E., Jennings, R.B., Katz, A.M., and Morgan, H.E., eds.) Raven Press, New York.). Of the numerous pathologic features of spontaneous hypertension, many represent compensatory mechanisms that offset the primary abnormality. With the high prevalence rate of hypertension and less than optimal treatment regimes available, an improved form of therapy is needed.

Chronic renal failure requiring renal dialysis is known to affect over 100,000 Americans and there is a large undiagnosed population with subclinical renal

failure (Nissenson, A.R., et al., 1991. Annals of Internal Medicine. 114:402-16). Many of these individuals suffer from anemia secondary to the loss of erythropoietin, a 34,000 MW glycoprotein that is primarily synthesized in the kidney. The anemia that results can result in significant clinical morbidity and these individuals may require multiple blood transfusions which can lead to severe iron overload, subsequent organ dysfunction and/or viral infection. Furthermore, anemia is a well known side-effect in AIDS patients undergoing AZT therapy as well as cancer patients undergoing chemotherapy (Kanz, L. A. et al., 1991, Am J Clin Oncol 14 (suppl. 1): S27-S33). A relatively new therapy involves the use of recombinant erythropoietin. Over 2000 anemic hemodialysis patients have been treated and even at low dose therapy there has been nearly a 100% response rate with complete resolution of the anemia in most patients. The drug can be administered subcutaneously or intravenously and is usually required three times a week. The use of gene therapy for administration of erythropoietin would be an improvement over existing technologies.

There are a large number of disorders that result in growth delay many of which may be treatable with growth hormone. This protein has been used therapeutically in the past for a limited number of disorders most of which are the result of primary growth hormone deficiency. Many of the early difficulties with growth hormone ("GH") therapy was the result of the paucity of the protein which was prepared from pooled human pituitary extracts. Furthermore, the protein on occasion was tainted with the Creutzfeldt-Jakob infectious agent which leads to a lethal disease. With the ability to produce this hormone by recombinant DNA techniques in 1985, growth hormone has been used successfully in clinical trials for a number of disorders that are not related to primary growth hormone deficiency. Some of these disorders include Turner's Syndrome (Reiter, JEC., M. Craven, G. Van-Vliet (1991),

Acta-Endocrinol. 125:38-42) which has an incidence of 1/2500 live female births and Noonan's syndrome (Ahmed, M.L., et al.,(1991), Acta Paediatrica Scadinavica 80:446-50). Growth hormone therapy may also be useful in children who exhibit growth failure secondary to treatment of chronic disease states such as leukemia, brain tumors or other malignancies (Papadimitriou, A., et al.,(1991). Archives of Disease in Childhood 66:689-92). Very recently growth hormone has been used successfully to increase lean body mass, decrease adipose-tissue mass and increase the lumbar bone density in normal men over the age of 60 years (Rudman, D, et al., (1990). N Engl. J, Med. 323:52-54). Thus, growth hormone may have an anti-aging effect and may be beneficial in slowing the aging process. A number of additional disorders that result in growth delay may also be amenable to growth hormone therapy.

PKU is an inherited deficiency of the hepatic enzyme phenylalanine hydroxylase ("PAH"). Approximately 1 in 12,000 newborns are effected with PKU which is characterized by elevated serum and tissue levels of phenylalanine which causes severe mental retardation. Dietary restriction of phenylalanine in childhood is effective in preventing mental retardation, though long term dietary restriction through late childhood and adulthood is nearly impossible, and most individuals suffer slow, progressive deterioration of cognitive function. Women who have elevated phenylalanine levels are also at risk for a syndrome of maternal PKU in which genetically normal children suffer teratogenic effects and mental retardation. It is generally believed that any therapy which lowered and maintained phenylalanine levels to below about 300 micromolar would prevent the pathological consequences of this disease (Harrison's Principle of Internal Medicine, twelfth edition, D.D. Wilson, E. Braunwald et al., eds. 1991, McGraw-Hill Inc., New York). Other enzymes have been identified to be

defective or deficient in inherited metabolic disorders include methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase.

Serum protein disorders are another group of diseases that are amenable to gene therapy treatment. Deficiencies in clotting factors are responsible for the group of diseases known as hemophilia. Hemophilia A is caused by a deficiency in active Factor VIII. Current therapy involves replacing these factors with protein isolated from blood donors or more recently the replacement of the defective protein with active proteins produced in bacteria by recombinant DNA technologies. The former method has occasionally resulted in the introduction of contaminating viruses, including HIV. Both methods require multiple injections of the proper factor.

Summary of the Invention

This invention features methods and compositions for treating diabetes mellitus, Parkinson's disease, cardiovascular disease, hypercholesterolemia, hypertension, anemia, thrombosis, growth disorders, metabolic disorders, such as PKU and serum protein disorders, such as hemophilia A, by administering nucleic acids capable of expression in an animal, e.g., a human, to replace or augment a particular defective or missing nucleic acid expression product. In some embodiments the present invention discloses compositions and methods for gene therapy directed at the liver for the treatment of some or all of the above set forth diseases.

In one embodiment of the present invention there is provided a method of transforming cells *in vivo* by either introducing a nucleic acid vector or a viral or retroviral vector. These vectors can be introduced directly into the liver or into the blood stream. In the latter case, the vectors may be attached to molecules which target the liver or other target organs. For *in vivo* introduction of

the vector the DNA transporter can be used. Alternatively, the vector can be combined with particles and administered. The particles may be selected from the group consisting of magnetically charged particles bound to DNA or particles for slow release of DNA. Another example is to use the gene gun of DuPont. (Yang, N.S., et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:9568-72). One skilled in the art will readily recognize these examples only reflect some of the variety of methods for *in vivo* transformation of cells and will pick the method most appropriate for their use. The term "hepatocyte" herein means a liver cell which is one of the essential elements of the liver and contains the functional aspects of liver function.

The term "retrovirus" as used herein refers to an RNA containing virus which replicates through a double strand of DNA intermediate. Once copied, the viral RNA integrates into the host chromosome and is subsequently replicated or transcribed much like a cellular gene. Retroviruses are used as vectors for introducing foreign genes or foreign nucleic acid into eukaryotic cells. Generally, the nucleic acid sequence of interest, is in the nucleic acid cassette. In the present invention the nucleic acid cassette is cloned downstream of a retroviral LTR in a vector lacking key retroviral genes, the product of which are supplied in trans by a defective helper virus. On entry into the host cell, the retroviral vector is reverse transcribed and can integrate into the host genome. Schematic representation of the retroviral viruses which are useful in the present invention are shown in Figures 1 and 11.

The term "adenovirus" refers to a small non-enveloped double-stranded DNA virus capable of infecting non-dividing cells.

Another embodiment of the present invention provides for the treatment of a variety of diseases or conditions. This embodiment comprises the step of introducing a

transformed cell, such as a hepatocyte, into an animal. The transformed cell contains a nucleic acid vector containing a nucleic acid fragment encoding for the expression of a protein or polypeptide. The vector is capable of expressing the protein or polypeptide in the transformed cell.

In an alternate embodiment the disease or condition is treated by *in vivo* formation of a transformed cell, such as a hepatocyte, by introducing a nucleic acid vector or viral vector into the cell. The vector contains a nucleic acid vector containing a nucleic acid fragment encoding the sequence for a protein or polypeptide. The vector is capable of expressing the protein in the transformed cell.

In one embodiment the present invention provides a method of treating diabetes mellitus by transforming cells, such as hepatocytes, *in vivo* or *in vitro* with a vector containing a nucleic acid fragment encoding a functional insulin molecule or a proinsulin molecule, which may be modified intracellularly to produce a functional insulin molecule, and administering the transformed cells to an animal, thereby elevating levels of insulin in the treated animals.

In another embodiment of the present invention there is provided an *in vivo* or *in vitro* method of treating Parkinson's disease by administering a nucleic acid sequence permitting the expression of tyrosine hydroxylase ("TH").

In another embodiment of the present invention there is provided an *in vivo* or *in vitro* method for treating cardiovascular disease by the production of a constitutive or regulated amount of protein for the treatment of cardiovascular disease. In the treatment of cardiovascular disease, the protein is selected from a group consisting of apolipoprotein A1 ("apo A1"), lipoprotein lipase ("LPL"), apolipoprotein E ("apo E"),

cholesterol-7α-hydroxylase ("C7αH") and combinations thereof.

In another embodiment of the present invention there is provided an *in vivo* or *in vitro* method for treating hypercholesterolemia by the production of a constitutive or regulated amount of an LDL receptor.

In another embodiment of the present invention there is provided an *in vivo* or *in vitro* method of treating hypertension. For the treatment of hypertension, the cell, such as a hepatocyte expresses the atrial natriuretic factor ("ANF") or γMSH.

In another embodiment of the present invention there is provided an *in vivo* or *in vitro* method of treating anemia by administering a nucleic acid sequence permitting the expression of erythropoietin ("EPO").

In another embodiment of the present invention there is provided an *in vivo* or *in vitro* method for treating thrombosis by the production of a constitutive or regulated amount of protein for the treatment of thrombosis. In the treatment of thrombotic disease, the protein is selected from the group consisting of thrombomodulin, protein C, protein S, and antithrombin-III and combinations thereof.

In another embodiment of the present invention, an administered nucleic acid sequence encodes a growth hormone for the treatment of diseases of growth.

In another embodiment of the present invention there is provided an *in vivo* or *in vitro* method for treating metabolic disorders, such as phenylketonuria, by the production of a constitutive or regulated amount of protein for the treatment of metabolic disorders. In the treatment of metabolic disorders, the protein is selected from the group consisting of PAH, methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase. By "metabolic or metabolism" is meant the sum of all the physical and

chemical processes by which living cells produce and maintain themselves and by which energy is made available for the use of an organism. By "metabolic disorder" is meant a defect in a metabolic pathway, usually catalyzed by an enzyme. By "metabolic pathway" is meant a series of stepwise biochemical changes in the conversion of some precursor substance to an end product.

In another embodiment of the present invention, an administered nucleic acid sequence encodes a clotting factor, such as Factor VIII for the treatment of serum protein disorders, such as hemophilia A. By "serum protein disorder" is meant a genetic defect or disease caused by a missing or defective protein or nucleic acid which results in abnormalities in blood clotting.

"Treating" means elevating the level of insulin from an abnormally decreased level closer to a normal range of levels or if appropriate exceeding normal levels, or that the detrimental health effects of an abnormally decreased level of insulin may be diminished or abolished.

"Treating" also means elevating the level of one or more of apo A1, LPL, apo E, C7αH from an abnormally decreased level closer to a normal range of levels, or if appropriate exceeding normal levels, or that the detrimental health effects of an abnormally decreased level of at least one of these molecules may be diminished or abolished.

"Treating" also means elevating the level of one or more of thrombomodulin, protein C, protein S or antithrombin-III from an abnormally decreased level closer to a normal range of levels, or if appropriate exceeding normal levels, or that the detrimental health effects of an abnormally decreased level of at least one of these molecules may be diminished or abolished.

"Treating" also means elevating the level of one or more of PAH, methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase from an

abnormally decreased level closer to a normal range of levels, or if appropriate exceeding normal levels, or that the detrimental health effects of an abnormally decreased level of at least one of these molecules may be diminished or abolished.

"Treating" also means elevating the level of LDL receptor, ANF, γMSH, EPO, TH, growth hormone and Factor VII from an abnormally decreased level closer to a normal range of levels, or if appropriate exceeding normal levels, or that the detrimental health effects of an abnormally decreased level of at least one of these molecules may be diminished or abolished.

Any one of the insulin, proinsulin, TH, apo A1, LPL, apo E, C7αH, LDL receptor, ANF, γMSH, EPO, thrombomodulin, protein C, protein S, antithrombin-III, growth hormone, Factor VIII and PAH, methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase (hereinafter "the therapeutic molecules") may be expressed only in a specific cell, such as a liver cell (hepatocyte), in which case "elevated" is relative to the normal range, which may include zero expression, of the therapeutic molecules expressed in such liver cells of a particular species or individual within that species. Alternatively or concurrently, any one of the therapeutic molecules can be expressed in the blood circulation or blood stream of an animal and in that case "elevated" is relative to the normal range of the particular therapeutic molecule typically expressed in the blood stream of a particular species or individual within that species.

The term "elevating" means that for a given one of the therapeutic molecules a level above those normally found in such an animal will result from the administration of nucleic acid vectors encoding a therapeutic molecule through the expression of a nucleic acid sequence contained in the vector.

In a preferred embodiment, an isolated nucleic acid encoding at least one of the therapeutic molecules, is used to elevate the level of such a molecule within an animal or a cell. Such isolated nucleic acid sequences include a cDNA, genomic DNA clone, RNA or an mRNA species which encode a sequence of one of the therapeutic molecules obtained from an animal and exhibiting some or all of the functional characteristics associated with such a therapeutic molecule. Further, any such nucleic acid sequence which encodes a portion of one of the therapeutic molecules which exhibits the functional characteristics of the therapeutic molecule as known in the art are within the preferred embodiment.

A functional therapeutic molecule may encompass any part of the therapeutic molecule, alone or as part of a fusion protein, *e.g.*, an insulin molecule linked through a chemical bond to a part or whole of another protein, so long as it is functional.

Generally, such nucleic acid encoding at least one of the therapeutic molecules will have homology to known genomic or cDNA sequences from human or other species. By homology is meant any one of the therapeutic molecules will have at least about 70% sequence identity along the length of the nucleic acid, preferably at least 80%, 90% or even 100% identity for at least 100, 200 or all nucleotide bases.

In a preferred embodiment, the isolated nucleic acid sequence encodes any one of the therapeutic molecules including additions, deletions or modifications to some or all of the sequence of the nucleic acid. That is, the nucleic acid sequences may be altered at its 5′ end 3′ end, or at any point intermediate to the 5′ or 3′ ends of the sequence.

By "modification" is meant that nucleic acid base analogues as are known in the art may be present, or that one base, for example adenine may be substituted for another base, for example, guanine; the phosphodiester

linkage may be modified as is known in the art, for example by substitution of a thioester linkage; or the sugar moiety of the nucleic acid may be modified as is known in the art, for example, substitution of 2'-deoxyribose with ribose or substitution of ribose with 2'-deoxyribose. These modifications may be made to one or more bases in the nucleic acid sequence. Modifications also include changes which, for example, stabilize the nucleic acid, but do not effect the function of the therapeutic molecule (as can be determined by routine testing). Additionally, protein sequences comprising less than or more than a whole therapeutic molecule but which are nevertheless functional, may be mapped by mutational analysis or various clones may be created and the activity of proteins expressed from such clones assayed or other routine testing as is known in the art may be utilized.

In another preferred embodiment, the present invention includes a nucleic acid sequence which will specifically hybridize to a segment of the nucleic acid sequence of at least one of the therapeutic molecules. This includes nucleic acid sequences which hybridize to any segment of one of the therapeutic molecules in a manner which is indicative of specific binding as opposed to non-specific background binding under conditions of stringency which would decrease non-specific binding, but would not be considered highly stringent.

In a preferred embodiment the invention includes a nucleic acid sequence which will only hybridize to a segment of the nucleic acid sequence of one of the therapeutic molecules under highly stringent conditions. By "highly stringent conditions" is meant that non-specific hybridization would be expected to occur at a very low rate, *e.g.*, hybridization would not be expected if there is more than about one nucleic acid base mismatch per 20 nucleotide bases of any one of the therapeutic molecule nucleic acid sequence.

In another preferred embodiment, a nucleic acid sequence encoding any one of the therapeutic molecules is provided within a vector. The term "vector" as used herein refers to a nucleic acid, *e.g.*, DNA derived from a plasmid, cosmid, phagemid or bacteriophage, into which fragments of nucleic acid may be inserted or cloned. The vector can contain one or more unique restriction sites for this purpose, and may be capable of autonomous replication in a defined host or organism such that the cloned sequence is reproduced. The vector molecule can confer some well-defined phenotype on the host organism which is either selectable or readily detected. Some components of a vector may be a DNA molecule further incorporating a DNA sequence encoding a therapeutic or desired product, and regulatory elements for transcription, translation, RNA stability and replication. A viral vector in this sense is one that contains a portion of a viral genome, *e.g.*, a packaging signal, and is not merely DNA or a located gene within a viral article. The term "therapeutic molecule vector" is synonymous with the above-recited definition.

With respect to vectors, the pharmacological dose of a vector and the level of gene expression in the appropriate cell type includes sufficient protein or RNA to increase the level of protein production.

The use of formulated nucleic acid expression vectors for systemic administration has a significant advantage in that a tissue-specific promoter can be incorporated such that the therapeutic gene product is produced only in selected cells, even if the vector is distributed elsewhere, thus restricting the biological effect of the vector to the desired target. A "formulated nucleic acid vector" is a nucleic acid vector associated with any of the formulations set forth below.

A preferred means for delivering the vector to the target cell involves the use of formulations for delivery to the target cell in which a purified vector is

associated with elements such as lipids, proteins, carbohydrates, synthetic organic compounds, or inorganic compounds which enhance the entry of the vector into the nucleus of the target cell where gene expression may occur. The term "formulation" as used herein refers to non-genetic material combined with the vector in a solution, suspension, or colloid which enhances the delivery of the vector to a tissue, uptake by cells within the tissue, intracellular trafficking through the membrane, endosome or cytoplasm into the nucleus, the stability of the vector in extracellular or intracellular compartments, and/or expression of genetic material by the cell. This includes formulation elements enhancing the delivery, uptake, stability, and/or expression of genetic material into cells as well as the storage and packaging of nucleic acid. In a preferred embodiments of the present invention the vector and formulation comprises a nanoparticle which is administered as a suspension or colloid. The formulation may include lipids, proteins, carbohydrates, synthetic organic compounds, or in-organic compounds. Examples of elements which are included in a formulation are lipids capable of forming liposomes, cationic lipids, hydrophilic polymers, polycations (e.g. protamine, polybrine, spermidine, polylysine), peptide or synthetic ligand recognizing receptors on the surface of the target cells, peptide or synthetic ligand capable of inducing endosomal-lysis, peptide or synthetic ligand capable of targeting materials to the nucleus, peptide or synthetic ligands with fusogenic proteins, gels, slow release matrices, salts, carbohydrates, nutrients, or soluble or insoluble particles as well as analogues or derivatives of such elements. Ligands may be proteins, natural products, synthetic organic products, nutrients, drugs, hormones and may be bound covalently or non-covalently to nucleic acid or bound covalently or non-covalently to intermediate elements which binds nucleic acid. Examples of intermediate elements include

cationic moieties, intercalating compounds, or compounds that recognize chemically modified nucleic acid residues.

The methods of use of the herein-described vectors comprises the steps of administering an effective amount of the vectors to a human, animal or tissue culture. The term "administering" or "administration" as used herein refers to the route of introduction of a vector or carrier of nucleic acid into the body. The vectors of the above methods and the methods discussed below may be administered by various routes. Administration can be directly to a target tissue or may involve targeted delivery after systemic administration.

The term "delivery" as used herein refers to the process by which the vector comes into contact with the preferred target cell after administration. Administration may involve needle injection into cells, tissues, fluid spaces, or blood vessels, hypospray, electroporation, transfection, hypospray, iontophoresis, particle bombardment, or transplantation of cells genetically modified *ex vivo*. Examples of administration may, include intravenous, intramuscular, aerosol, oral, ocular systemic, intraperitoneal and/or intrathecal.

The term "target cell" or "target tissue" as used herein refers to the cell which is intended to take up the vector and express the therapeutic product. The therapeutic product may be active within the target cell, may be secreted from the target cell and exert its therapeutic effect on other cells within the target tissue, or may be secreted from the target cell into the general circulation for therapeutic effect on other tissues.

There is provided with this invention methods for targeted delivery of the nucleic acid vector to certain tissues based on the characteristics of the formulated particle and ligands capable of enhancing the association of the particle with certain target cells.

The term "uptake" as used herein refers to the translocation of the vector from the extracellular to intracellular compartments specifically the nucleus. This can involve receptor mediated processes, fusion with cell membranes, endocytosis, potocytosis, pinocytosis, etc. The vector may be taken up by itself with various elements of the formulation still bound to the vector.

There is described in this invention the application of certain methods for enhancing the uptake of the nucleic acid-vector into the target cell. These involve elements of the formulation which enable penetration of cellular membranes by fusogenic lipids or proteins, the action of ligands or drugs capable of enhancing endocytosis or penetration of the cellular membrane, the action of ligands capable of enhancing release of the nucleic acid-vector from the endosome, or the action of ligands capable of enhancing entry of the nucleic acid-vector into the nucleus.

Expression of a therapeutic gene product which may be active in the cell and tissue which takes up the nucleic acid, a therapeutic gene product which may be active on cells and tissue in the immediate proximity of the cell taking up the nucleic acid, or a gene product which may circulate throughout the body for action on cell and tissue at distant sites. In specific examples the gene products is a protein or RNA molecule.

The purpose of the vector is to provide expression of a nucleic acid sequence in tissue. Expression products may be proteins (including but not limited to pure protein (polypeptide), glycoprotein, lipoprotein, phosphoprotein, nucleoprotein, etc.) or RNA. The nucleic acid sequence is contained in a nucleic acid cassette. Expression of the nucleic acid can be continuous or controlled by endogenous or exogenous stimuli.

The term "nucleic acid cassette" as used herein refers to the genetic material of interest which codes for a protein or RNA. The nucleic acid cassette is positionally

and sequentially oriented within the vector such that the nucleic acid in the cassette can be transcribed into RNA, and when necessary, translated into a protein in the transformed tissue or cell. Preferably, the cassette has 3′ and 5′ ends adapted for ready insertion into a vector, e.g., it has restriction endonuclease sites at each end.

A variety of proteins can be encoded by the sequence in a nucleic acid cassette in the transformed tissue or cell. Those proteins which can be expressed may be located in the cytoplasm, nucleus, membranes (including the plasmalemma, nuclear membrane, endoplasmic reticulum or other internal membrane compartments), in organelles (including the mitochondria, peroxisome, lysosome, endosome or other organelles), or secreted.

The term "promoter" as used herein refers to a recognition site of a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibitory sequences termed "silencers".

The promoter can be one which is naturally (*i.e.*, as it occurs with a cell *in vivo*) or non-naturally associated with a the 5′ flanking region of a particular gene. Promoters can be derived from eukaryotic genomes, viral genomes, or synthetic sequences. Promoters can be selected to be non-specific (active in all tissues), tissue specific, regulated by natural regulatory processes, regulated by exogenously applied drugs, or regulated by specific physiological states such as those promoters which are activated during an acute phase response or those which are activated only in replicating cells. Non-limiting examples of promoters in the present invention include the retroviral LTR promoter, cytomegalovirus immediate early promoter, SV40 promoter, dihydrofolate reductase promoter. The promoter can also

be selected from those shown to specifically express in the select cell types.

One skilled in the art will recognize that the selection of the promoter will depend on the vector, the nucleic acid cassette, the cell type to be targeted, and the desired biological effect. One skilled in the art will also recognize that in the selection of a promoter the parameters can include: achieving sufficiently high levels of gene expression to achieve a physiological effect; maintaining a critical level of gene expression; achieving temporal regulation of gene expression; achieving cell type specific expression; achieving pharmacological regulation of gene expression. Any given set of selection requirements will depend on the conditions but can be readily determined once the specific requirements are determined.

The term "intron" as used herein refers to a section of DNA occurring in a transcribed portion of a gene which does not code for an amino acid in the gene product. RNA transcribed by RNA polymerase as it reads through the intron is included in a precursor RNA, and is then excised so that it is not present in the mature messenger RNA nor translated into protein). The term "exon" as used herein refers to a portion of a gene that is included in the transcript of a gene and survives processing of the RNA in the cell to become part of a messenger RNA (mRNA). The intron/exon boundary will be that portion in a particular gene where an intron section connects to an exon position. The terms "TATA box" and "CAP site" are used as they are recognized in the art.

In the present invention, the nucleic acid cassette in the retrovirus vector or the DNA vector is activated with a promoter. The promoter used can be any of a wide variety of promoters known in the art. Examples of these promoters are the cytomegalovirus (CMV) promoter, phosphoglycerol kinase (PGK) promoter, albumin promoter, phosphoenolpyruvate carboxykinase (PEPCK) promoter,

pyruvate kinase (PK) promoter, spot 14 glucose induced protein (P14) promoter, P-450 promoter, C reactive promoter, C/EBP promoter and transferrin promoter.

One skilled in the art will readily recognize that the selection of the promoter will depend on the vector, the cell type, the nucleic acid cassette and the desired biological effect. In the selection of a promoter, the parameters can include achieving sufficiently high levels of nucleic acid expression to achieve a physiological effect; maintaining a critical steady state of nucleic acid expression; achieving temporal regulation of nucleic acid expression; achieving tissue specific expression of nucleic acid. Any given set of selection requirements will depend on the conditions, but one skilled in the art can readily determine the conditions once the specific requirements are determined. Those promoters which are naturally occurring in the hepatocyte or other cell which may be transformed or which are naturally used in the regulation of proteins involved in the present invention are preferred.

It should be noted that the promoter can regulate the type of expression seen in the present invention. Selection of the promoter and the regulatory elements can result in constitutive production of the protein, that is, there is a steady state level of expression of the quantity of the protein; or it can result in regulatory production, that is, the protein can be regulated and the amount of protein can be responsive to the environmental factors.

When it is required to produce a regulated amount of a protein the transformed hepatocyte or other selected cell types, are capable of regulated production of the protein. The cell such as a hepatocyte, thus, produces a level of protein in response to regulating molecules. For example, in the case of proinsulin and insulin, the amount of proinsulin or insulin which is expressed by the cell such as a hepatocyte can be regulated by fluctuations in the amount of regulatory molecules — for example glucose

or blood insulin. This is achieved by using the regulatory mechanism native to the transformed cell, such as a hepatocyte or incorporating appropriate promoters and appropriate regulatory elements within a vector or multiple vectors.

Further, when using the LTR promoters, the repeat sequences can be replaced with a cell specific enhancer. Examples of the cell specific enhancers which are used are the phosphoglycerol kinase, C/EBP, transferrin and albumin enhancers.

Any of a variety of viruses can be used, including retrovirus, adenovirus, adeno-associated virus, vaccinia virus and herpes simplex virus.

It is also known to those skilled in the art that one can enhance the effect of the cell such as a hepatocyte and/or facilitate the incorporation of the nucleic acid vector into the cell such as a hepatocyte. This is achieved by the addition of growth promoting factors with either the viral or DNA vectors. As used herein the term "growth promoting factor" refers to those factors which (1) enhance or facilitate growth of the cell and/or (2) enhance uptake and incorporation of the viral or DNA vector into the cell. One skilled in the art will readily recognize there are a variety of growth promoting factors which are helpful in achieving these goals. In the present invention, it is found that a variety of growth factors including hepatocyte growth factor, epidermal growth factor, TGF-β and growth promoting drugs are helpful.

In the present invention, one method of making the transformed cells incorporating the nucleic acid cassette is the *ex vivo* approach. This approach includes the steps of harvesting cells such as hepatocytes, cultivating the cells, transducing or transfecting the cells and selecting the transformed cells of interest. In this procedure, a selective marker can also be added to the vectors in order to enhance the ability to select the transformed cells.

The cells can be garnered in a variety of ways. They can be taken from the individual who is to be later injected with the cells that have been transformed or they can be collected from other sources, transformed and then injected into the individual of interest.

Once the *ex vivo* modified and transformed cell is collected, it can implanted into an animal for treatment. This method comprises the steps of contacting the cell such as a hepatocyte with media containing the infectious recombinant virus vector or DNA vector and maintaining the cultured cells in the media for sufficient time and under conditions appropriate for uptake and transformation of the cells by the viral vector or DNA vector.

The cells can be introduced into an orthotopic location (the body of the liver) or heterotopic locations by injection of cell suspensions into tissues. One skilled in the art will recognize that the cell suspension may contain: salts, buffers or nutrients to main viability of the cells; proteins to ensure cell stability; and factors to promote angiogenesis and growth of the implanted cells.

In an alternative method, cells such as hepatocytes may be grown *ex vivo* on a matrix consisting of plastics, fibers or gelatinous materials which can be surgically implanted in an orthotopic or heterotopic location. This matrix may be impregnated with factors to promote angiogenesis and growth of the implanted cells.

In a preferred embodiment, a vector comprising nucleic acid encodes any one of the therapeutic molecules, wherein the therapeutic molecule vector, e.g., an insulin vector, a proinsulin vector, an apo A4 vector, an LPL vector, an apo E vector, a C7αH vector, a thrombomodulin vector, a protein C vector, a protein S vector, an antithrombin III vector, an EPO vector, a TH vector, an ANF vector, an γMSH vector, a growth hormone vector, an LDL receptor vector, a Factor VIII vector, a PAH vector, a methylmalonyl CoA mutase vector, a proprionyl CoA carboxylase vector, an ornithine transcarbamylase vector, a carbamylphosphate

synthetase vector and an arginase-arginosuccinate lyase vector is adapted to cause expression of one of the therapeutic molecules. Expression includes the efficient transcription of an inserted gene or nucleic acid sequence within the vector. Expression products may be proteins, polypeptides or RNA. By "expression of a therapeutic molecule" is meant that a complete or partial functional therapeutic protein molecule is produced from the vector containing the nucleic acid encoding the therapeutic molecule.

In another preferred embodiment, a vector having nucleic acid sequences encoding one of the therapeutic molecules is provided in which the nucleic acid sequence is expressed only in specific tissue. That is, a complete or partial functional therapeutic molecule is produced from the vector containing the nucleic acid encoding the therapeutic molecule only in one or more predetermined, desired tissues. For example, muscle only, or liver only, or muscle and liver only.

In a preferred embodiment, a vector for the expression of one of the therapeutic molecules nucleic acid sequence has a tissue-specific promoter, a therapeutic molecule encoding nucleic acid sequence, and a post-transcriptional processing control sequence. The term "tissue-specific promoter" means that the promoter will allow transcription of RNA from the vector primarily only in a specific tissue in which the promoter is activated. For example, muscle cell specific promoters will only allow transcription in muscle cells or liver cell specific promoters will only allow transcription in liver cells. However, even with tissue-specific promoters some low level (about 10% or less than that observed in the desired tissue) expression might occur in other cell types. The promoter would still be defined as tissue specific. Methods of achieving tissue-specific gene expression as set forth in International Publication No. WO 93/09236, filed November 3, 1992 and published May 13, 1993; International Application No.

PCT/US93/03993, filed April 28, 1993; International Application No. PCT/US93/03985, filed April 28, 1993; and U.S. patent application entitled "Specific Expression Vectors and Methods of Use", filed November 1, 1993 and U.S. patent application entitled "Keratin K1 Expression Vectors and Methods of Use"; all (including drawings) hereby incorporated by reference herein. By "post-transcriptional processing control sequence" is meant, for example, sequences which control intron deletion and exon splicing, polyadenylation or other modifications affecting RNA stability or RNA transport to cellular locations.

In another embodiment of the invention a method of modifying a consensus sequence cleavage site of a protein is provided so that the consensus sequence cleavage site may be cleaved by a non-native peptidase. By "non-native peptidase" is meant a peptidase which would not cleave the protein at the consensus sequence prior to its modification. For example, a protein which is only cleaved in β-pancreatic cells by native peptidases, upon modification could be cleaved in a hepatic cell by non-native peptidases.

In a preferred embodiment the nucleic acid encoding the proinsulin molecule is modified to alter the cleavage sites, through which proinsulin is converted to insulin, so that natural liver endopeptidases, as opposed to pancreatic peptidases, cleave the proinsulin into insulin. The proinsulin nucleic acid molecule is modified at a consensus sequence flanking the boundary between the A polypeptide and C polypeptide of proinsulin. This modification encompasses those changes in the amino acid sequence which allow any peptidase to cleave proinsulin between the A and C. Further, the consensus sequence between the B and C chain may also be altered to allow for cleavage by any peptidase. Modifications of the A/C and A/B consensus sequences which confer cleavability by non-native peptidases may be screened for by functional insulin assays as are known in the art. Additionally,

modified amino acids, as are known in the art, may be used in the creation of a modified consensus sequence. In a preferred embodiment the A/C consensus sequence is modified to allow it to be cleaved by type I convertase.

Yet an additional preferred embodiment, comprises a cell stably transfected with a therapeutic molecule vector. The term "transfected" as used herein refers to a cell having undergone the process of introduction of nucleic acid or a nucleic acid vector into a cell. Various methods of transfecting a cell are possible including microinjection, $CaPO_4$ precipitation, lipofection (liposome fusion), electroporation and use of a gene gun. The term "stable" as used herein refers to the introduction of a gene into the chromosome of the targeted cell where it integrates and becomes a permanent component of the genetic material in that cell. An episomal transfection is a variant of stable transfection in which the introduced gene is not incorporated in the host cell chromosomes but rather is replicated as an extrachromosomal element. This can lead to apparently stable transfection of the characteristics of a cell.

A cell may be co-transfected with a vector containing a selectable marker. This selectable marker is used to select those cells which have become transfected. Types of selectable markers which may be used are well known to those of ordinary skill in the art.

In still another preferred embodiment, there is provided a transfected cell wherein one of the therapeutic molecules is expressed as a secreted protein. By "secreted protein" is meant a protein which is not associated with the cell membrane, but rather is intracellularly processed for secretion into the extracellular environment or other cellular compartment.

Alternatively, a transfected cell containing a therapeutic molecule may only be transiently transfected, resulting in transient expression of the therapeutic molecule. The term "transient" as used herein relates to

the introduction of a gene into a cell to express a therapeutic molecule, where the introduced gene is not integrated into the host cell genome and is accordingly eliminated from the cell over a period of time. Transient expression relates to the expression of a gene product during a period of transient transfection.

In yet a further preferred embodiment, there is provided a cell stably transformed with a therapeutic molecule. The term "transformed" as used herein refers to a process or mechanism of inducing transient or permanent changes in the characteristics (expressed phenotype) of a cell by the mechanism of gene transfer whereby DNA or RNA is introduced into a cell in a form where it expresses a specific gene product or alters the expression or effect of endogenous gene products. The term "stable" as used herein refers to the introduction of gene(s) into the chromosome of the targeted cell where it integrates and becomes a permanent component of the genetic material in that cell. Gene expression after stable transformation can permanently alter the characteristics of the cell leading to stable transformation. An episomal transformation is a variant of stable transformation in which the introduced gene is not incorporated in the host cell chromosomes but rather is replicated as an extrachromosomal element. This can lead to apparently stable transformation of the characteristics of a cell.

Cells may be co-transformed with a vector containing a selectable marker. This selectable marker is used to select those cells which have become transformed. Types of selectable markers which may be used are well known to those of ordinary skill in the art.

The embodiments and definitions set forth above with respect to transfected cells, relating to cell surface proteins and secreted proteins, are equally applicable to a therapeutic molecule expressed in stably transformed cells.

A cell transformed with a therapeutic molecule vector may only be transiently transformed, resulting in transient expression of a therapeutic molecule. The term "transient" as used in transiently transformed is identical to that set forth with respect to transfected cells.

An additional preferred embodiment, provides for a transgenic animal containing a therapeutic molecule vector. By "transgenic animal" is meant an animal whose genome contains an additional copy or copies of the gene from the same species or it contains the gene or genes of another species, such as a gene encoding a therapeutic molecule introduced by genetic manipulation or cloning techniques, as described herein and as known in the art. The transgenic animal can include the resulting animal in which the vector has been inserted into the embryo from which the animal developed or any progeny of that animal. The term "progeny" as used herein includes direct progeny of the transgenic animal as well as any progeny of succeeding progeny. Thus, one skilled in the art will readily recognize that if two different transgenic animals have been made each utilizing a different gene or genes and they are mated, the possibility exists that some of the resulting progeny will contain two or more introduced genes. One skilled in the art will readily recognize that by controlling the matings, transgenic animals containing multiple introduced genes can be made.

In another preferred embodiment, a method of introducing a continuous supply of a therapeutic molecule into an animal or a tissue culture by administering an effective amount of a vector is provided. By "continuous" is meant that the therapeutic molecule is constitutively expressed without the need for the addition of an exogenously administered activating compound to initiate expression. The term "effective amount" means an amount sufficient to give expression of some amount of a

therapeutic molecule in an *in vivo* tissue or tissue culture.

In another preferred embodiment, a method of gene replacement is set forth. "Gene replacement" as used herein means supplying a nucleic acid sequence which is capable of being expressed *in vivo* in an animal and thereby providing or augmenting the function of an endogenous gene which is missing or defective in the animal.

In another preferred embodiment, an *in vivo* method of administering a nucleic acid sequence is provided, as described below. In a further preferred embodiment, naked DNA may be administered. The term "naked DNA" means substantially pure DNA which is not associated with protein, lipid, carbohydrate or contained within a cell or an artificial delivery system such as a liposome. A tissue or cell may also be transduced with a therapeutic molecule vector. The term "transduced or transduction" as used herein refers to the process of introducing a recombinant virus into a cell by infecting the cell with the virus particle. The virus may be administered substantially simultaneously, *i.e.*, the therapeutic molecule nucleic acid sequence and the virus may be administered in the same composition or that the administration of one may follow the other by about up to one hour.

In all of the preceding vectors set forth above, a further aspect of the invention is that the nucleic acid sequence contained in the vector may include additions, deletions or modifications to some or all of the sequence of the nucleic acid, as defined above.

In specific embodiments of the *in vivo* and *in vitro* method for the treatment of disease, such as those set forth above, a vector is selected such that it can produce either a constitutive level, that is constant level, of the protein or the vector may include regulatory elements

such that a regulated amount of a protein encoded by a nucleic acid contained in the vector can be expressed.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments in the invention.

Detailed Description of the Invention

The drawings will first briefly be described.

Drawings:

Figure 1 illustrates production of a recombinant retrovirus encoding the rat 1 insulin gene. PCR was performed to rescue the 5' end of the rat insulin 1 cDNA described by Ullrich et al, and to add a consensus Kozak sequence. The PCR product was blunt cloned into the Sma 1 site of SK Bluescript, sequenced, excised with Hind III/ BamH1 (blunt-ended with Klenow) and cloned into the retroviral plasmid LNCX after Hind III/ Hpa 1 digestion. The CMV and Neo genes were removed by digestion with Bcl 1 and Hind III (blunt-ended with Klenow) and ligated. The resulting plasmid LX/rINS, encoded the 5' LTR, the rat insulin gene, and the 3' LTR. This plasmid was transfected into retroviral packaging cell line GPAM-12, 30 colonies were isolated, and assayed for retroviral production by transducing rat embryo fibroblast 208F cell line. RIA for rat insulin was performed on the supernatants and clone 28, being a high expresser, was used for further studies.

Figure 1B represents the results of n=4 independent transduction experiments with clone 28, n=2 media controls and n=2 LX/hAAT transduction experiments. The titre of clone 28 was determined by centrifuging 30 mls of supernatant from LX/rINS and LX/β-Geo over a 30% sucrose cushion, the RNA was isolated, dot blotted, probed with LTR sequence. A beta counter determine the ratio of LX/rINS to LX/β-Geo. Since the titre of LX/β-Geo was 1 X 10^6 PFU/ml by X-gal staining, the apparent titre of LX/rINS is 1 X 10^6 PFU/ml.

Figure 2 illustrates the effect of streptozotocin treatment in experimental rats. Panel A: Percent weight change in rats following treatment with streptozotocin. All rats were weighed and fasted for 6 hours, then streptozotocin, at a dose of 250 mg/Kg was dispensed for each cage of three rats. The streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5) immediately before intraperitoneal injection. Rats were weighed at the indicated times, and their percent change in weight from day 0 (the days of the streptozotocin treatment) was calculated. Each point is the average ± standard deviation. There are 8 rats in the media treatment group (closed squares), 10 rats in the LX/hAAT treatment group (open triangles) and 16 rats in the LX/rINS treatment group (open squares). Panel B: Survival curves of the animals in the three treatment groups.

Figure 3 illustrates serum chemistry analyses of rats following treatment with streptozotcin. Blood was collected by retro orbital bleeding, allowed to clot on ice for 30 minutes, followed by centrifugation at 10,000 X g for 15 minutes at 4°C. Panel A: Serum ketones were detected by spotting 10-15 μl of serum on an Ames Ketostix. Panel B: Aspartate aminotransferase (AST) activity. Panel C: Serum creatinine levels. AST, ALT, LDH, BUN and creatinine levels were determined by a Boehringer Mannheim/Hitachi Model 704 Automated Serum Chemistry Analyzer.

Figure 4 illustrates serum insulin concentrations (Panel A) and serum glucagon concentrations (Panel B) in the three animal treatment groups. Blood was collected and 4.0 mM EDTA was added as a protease inhibitor. Serum was isolated by centrifugation as above. The samples were then frozen at -20°C and sent on dry ice via over night courier to LINCO Research, Inc. Immunoassay Services for radioimmunoassay analysis. Each bar represents mean ± standard deviation for media (n=8), LX/hAAT (n=10) and LX/rINS (n=16).

Figure 5 illustrates 24 hour blood glucose levels in the experimental animals were determined at various time points using a ONE TOUCH meter (LifeScan). Since it was expected that the rats in the control groups would succumb to diabetes and since there was no observed difference between rats treated with LX/hAAT and media in the previous experiments, the blood glucose measurements for rats in these two groups were combined under the name "Control". Panel A: Control rats are rats transduced with LX/hAAT or media; Panel B: Blood glucose levels of rats transduced with LX/rINS. Open boxes represent non-fasting blood glucose levels in which food and water were provided ad libitum (determined 3 days after streptozotocin treatment), and open circles represent fasting blood glucose levels in which food was removed at 8:00 am. Determined 5 days after streptozotocin treatment. The results are mean ± standard deviation.

Figure 6 illustrates the effects of a recombinant adenovirus containing the rabbit low-density lipoprotein receptor ("rbLDLR") cDNA driven by the Rous sarcoma virus LTR promoter ("Adv/RSV-rbLDLR"). The function of this virus was tested *in vitro* by measuring the binding (□), uptake (■) and degradation (□) of [^{125}I]-labelled LDL in primary Watanabe hepatocytes transduced at different multiplicities of infection ("MOI"). Each point represents the mean ± standard deviation of three separate determinations from the same hepatocyte preparation.

Figure 7 illustrates total plasma cholesterol levels in Watanabe rabbits after infusion of $4x10^{11}$ viral particles/kg body weight of Adv/RSV-rbLDLR (■), Adv/RSV-hAAT (◆), or buffer (●) into the splenic vein. Each treatment group contained six age- and sex-matched animals. Multiple determinations were made prior to infusion to establish pretreatment levels. Blood samples were drawn twice weekly after infusion and total cholesterol levels were determined. By six days post-infusion, total cholesterol levels in the rabbits treated

with Adv/RSV-rbLDLR were significantly reduced ($p<0.001$) relative to those treated with Adv/RSV-hAAT or buffer. Standard errors are indicated for each data point.

Figure 8 illustrates plasma HDL-cholesterol levels in Watanabe rabbits after infusion of Adv/RSV-rbLDLR (■), Adv/RSV-hAAT (◆), or buffer (●). Each treatment group contained four age- and sex-matched animals. Multiple determinations were made prior to infusion to establish pretreatment levels. Blood samples were drawn twice weekly after infusion and HDL-cholesterol levels were determined. By ten days post-infusion, HDL-cholesterol levels in the rabbits treated with Adv/RSV-rbLDLR were significantly reduced ($p<0.01$) relative to those treated with Adv/RSV-hAAT or buffer. Standard errors are indicated for each data point.

Figure 9 illustrates plasma Apo AI levels in Watanabe rabbits after infusion of Adv/RSV-rbLDLR(■), Adv/RSV-hAAT (◆), or buffer (●). Each treatment group contained four age- and sex-matched animals. Multiple determinations were made prior to infusion to establish pretreatment levels. Blood samples were drawn twice weekly after infusion and HDL-cholesterol levels were determined. By ten days post-infusion, HDL-cholesterol levels in the rabbits treated with Adv/RSV-rbLDLR were significantly reduced ($p<0.005$) relative to those treated with Adv/RSV-hAAT or buffer. Standard errors are indicated for each data point.

Figure 10 illustrates the kinetics of the physiological responses in the Watanabe rabbits after rbLDLR gene delivery. The maximal increase in plasma HDL-cholesterol (■; N=4) occurred 3 to 4 days after the maximal decrease in plasma total cholesterol levels (□; N=6) in Watanabe rabbits treated with Adv/RSV-rbLDLR. Standard errors are indicated for each data point.

Figure 11 is diagrammatic representation of recombinant retroviruses.

The drawings are not necessarily to scale and certain features of the invention may be exaggerated in scale and shown in schematic form in the interest of clarity and conciseness.

Somatic gene therapy for a select number of disease has been attempted over the past decade. The first human gene therapy trials targeted cells of the immune system to restore immunologic function to individuals that suffer from severe combined immunodeficiency (Culver, K.W., et al., 1991, Hum Gen Ther 2:107-9). Additional studies have targeted the expression of recombinant genes to tumor infiltrating lymphocytes for the treatment of certain malignancies (Rosenberg, S.A., et al., 1990, N. Eng. J. Med. 323:570-8.). These therapies have relied on the use of *ex vivo* techniques were the patients cells are removed from the body, cultivated in the laboratory, transduced by viral vectors and then reimplanted into the patient.

Various other cell types have been considered potential targets for this type of gene therapy including fibroblast (Garver, R.I., Jr. 1987, Science 237:762-4; Axxelrod, J.H., 1990, Proc. Natl. Acad. Sci. USA 87:5173-7; Scharfmann, R., et al., 1990, Proc. Natl. Acad. Sci. USA 88:4626-30), myoblasts (Dhwawan, J., et al., 1991, Science 254:1509-12), thyroid follicular cells (O'Malley, B.W. Jr., et al., 1993, Otolaryn. Head Neck Surg. 108:51-62), epidermal cells (Morgan, J.R., et al., 1987, Science 237:1476-9), endothelial cells (Nabel, E.G., et al., 1989, Science 244:1342-4; Wilson, J.M., et al., 1989, Science 244:1344-6), and cells of the central nervous system (Kawaja, M.D., et al., 1992, J. Neurosci 12:2849-64; Chen, L.S., et al., 1991, J. Cell Biochem. 45:252-7). The success of these therapies has varied widely and few, if any, have clearly demonstrated their usefulness *in vivo*.

Recently, cultured hepatocytes have been proposed as targets for somatic gene therapy (Anderson, K.D., et al., 1989, Somat. Cell Mol. Genet. 15:215-27; Chowdhury, J.R.,

et al., 1991, Science 254:1802-5). The liver is an important target for gene therapies for treatment of metabolic disorders as well as therapies aimed at using the synthetic capacity of hepatocytes to secrete large amounts of therapeutic proteins into the blood. The large size of the liver, its ability to secrete large amounts of protein into the blood and its ability to perform various post-translational modifications required for the activity of certain gene products makes the liver an important target for such therapies (Ledley, F.D. 1993, Hepatology 18:1263-73).

In vivo gene therapy experiments directed at the liver have been reported for selected genes including: α1-antitrypsin (Kay M.A., et al., 1992, Proc. Natl. Acad. Sci. USA 89:89), Factor IX (Armentano D., et al., 1990, Prc. Natl. Acad. Sci USA 87:6141-5) LDL receptor (Wilson, J.M., et al., 1990, Proc. Natl. Acad. Sci. USA 87:8437-41), and methylmalonyl CoA mutase (Stankovics et al., 1992, Am. J. Hum. Genet. 51:A177). These therapies have varied widely in their ability to demonstrate successful treatment of disease.

Systemic administration of genes may be based on sterically-stabilized particles with the following characteristics: 1) prepared from cationic lipids for instance, sterically-stabilized with double-coating gangliosides, lipid derivatives of polyethylene glycol, 2) contain elements for enhanced uptake into target tissue such as specific ligands and elements for enhanced endosomal release and intracellular trafficking.

Hepatocytes represent achievable targets for intravenously administered gene therapeutic formulations. Hepatocyte delivery requires sterically stabilized particles which may gain access to the liver via its sinusoidal structure, ligands for hepatocyte receptors such to enhance uptake, and elements for enhancing endosomal release. Hepatocyte specific vectors can be used to control the level and location of gene expression

and can contain transcript stabilizers, regulatory elements, as well as elements for episomal replication. Of particular importance for treatment are disorders of lipid metabolism which lead to hyperlipidemia and atherogenesis. Hepatocyte-specific vectors and vector delivery technologies can provide controlled expression of at least one of the therapeutic molecules in hepatocytes for the treatment of various diseases. These technologies may also be employed for early entry into clinical trials of therapy for inborn errors of metabolism such as disorders or organic acid metabolism or phenylketonuria.

The clinical need is for products which can alter liver-specific pathways of intermediary metabolism including those involved in hyperlipidemia (hypercholesterolemia, hypertriglyceridemia) and inborn errors of metabolism (amino acid disorders, organic acid disorders). The most effective method of therapy requires a product which can be administered by non-invasive procedures, is non-immunogenic, has low toxicity, and can be administered repetitively. Gene therapy formulations for hyperlipidemia ideally have a prolonged duration of action and a low incidence of adverse effects to facilitate compliance. Gene therapy formulations with short duration may be applicable for metabolic disorders with high morbidity such as familial hypercholesterolemia or methylmalonic acidemia.

Progress in the development of new methods for the treatment of hypercholesterolemia and associated conditions has been greatly aided by the development of appropriate animal models. One such model, the Watanabe Heritable Hyperlipidemic Rabbit, has been available since the late 1970's (Watanabe, Y. 1980. *Atherosclerosis* 36:261-8). These animals bear a mutation in the LDL receptor gene that prevents the transport of the LDL receptor to the surface of hepatocytes, resulting in dramatic increases in plasma cholesterol levels. This rabbit model, together with LDL receptor knock-out mice

(Ishibashi, S., et al., 1993. *J. Clin. Invest.* 92:883-93) and other similar animal models, are now being used to determine the potential of various gene transfer methods for the treatment of hyperlipidemias (Wilson, J. M., et al., 1992. *Hum. Gene Ther.* 3:179-222; Wilson, J. M., et al., 1992. *J. Biol. Chem.* 267:963-7; Grossman, M., et al., 1992. *Hum. Gene Ther.* 3:501-10). DNA-based and retrovirus-mediated gene delivery has been attempted respectively *in vivo* and *ex vivo* in Watanabe rabbits (Wilson, J. M., et al., 1992. *Hum. Gene Ther.* 3:179-222; Wilson, J. M., et al., 1992. *J. Biol. Chem.* 267:963-67), and the *ex vivo* approach has also been examined in non-human primates (Grossman, M., et al., 1992. *Hum. Gene Ther.* 3:501-10). Thus far, the major problem with retrovirus-mediated approaches for treatment of hypercholesterolemia has been the low efficiency of therapeutic gene delivery to the target organs, and the requirement for dividing cells.

Administration

The nucleic acid sequence encoding at least one of the therapeutic molecules, can be administered to patients having a disease or condition characterized by an abnormal level of one of the therapeutic proteins *e.g.*, by exogenous delivery of the nucleic acid sequence encoding at least one of the therapeutic molecule as naked DNA, DNA associated with specific carriers, or in a nucleic acid expression vector to a desired tissue by means of an appropriate delivery vehicle, *e.g.*, a liposome, by use of iontophoresis, electroporation and other pharmacologically approved methods of delivery. Routes of administration may include intramuscular, intravenous, aerosol, oral (tablet or pill form), topical, systemic, ocular, as a suppository, intraperitoneal and/or intrathecal. The specific delivery route of a will depend on the use of the therapeutic molecule.

Some methods of delivery that may be used include:

a. encapsulation in liposomes,

b. transduction by retroviral, adenoviral or other viral vectors,

c. localization to nuclear compartment utilizing nuclear targeting site found on most nuclear proteins,

d. transfection of cells *ex vivo* with subsequent reimplantation or administration of the transfected cells,

e. a DNA transporter system.

At least three types of delivery strategies are useful in the present invention, including: Injection of naked therapeutic molecule DNA or charge modified naked therapeutic molecule DNA, particle carrier drug delivery vehicles which are also suitable for delivery of therapeutic molecule proteins, and retroviral or other viral expression vectors. Unmodified nucleic acid sequence encoding at least one of the therapeutic molecules, like most small molecules, are taken up by cells, albeit slowly. To enhance cellular uptake, the nucleic acid sequence encoding at least one of the therapeutic molecules may be modified in ways which reduce its charge but will maintain the expression of specific functional groups in the final translation product. This results in a molecule which is able to diffuse across the cell membrane, thus removing the permeability barrier.

Chemical modifications of the phosphate backbone will reduce the negative charge allowing free diffusion across the membrane. This principle has been successfully demonstrated for antisense DNA technology which shows that this is a feasible approach. In the body, maintenance of an external concentration will be necessary to drive the diffusion of the modified nucleic acid sequence encoding at least one of the therapeutic molecules into the cells of the tissue. Administration routes which allow the tissue to be exposed to a transient high concentration of the nucleic acid sequence encoding at least one of the therapeutic molecules, which is slowly dissipated by

systemic adsorption are preferred. Intravenous administration with a drug carrier designed to increase the circulation half-life of the nucleic acid sequence encoding at least one of the therapeutic molecules or at least one of the therapeutic molecule proteins can be used. The size and composition of the drug carrier restricts rapid clearance from the blood stream. The carrier, made to accumulate at the desired site of transfer, can protect the nucleic acid sequence encoding at least one of the therapeutic molecules from degradative processes.

Drug delivery vehicles are effective for both systemic and topical administration. They can be designed to serve as a slow release reservoir, or to deliver their contents directly to the target cell. An advantage of using direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been shown to increase the circulation half-life of drugs which would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

From this category of delivery systems, liposomes are preferred. Liposomes increase intracellular stability, increase uptake efficiency and improve biological activity.

Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver nucleic acids to cells and that the nucleic acid remains biologically active.

For example, a liposome delivery vehicle originally designed as a research tool, Lipofectin, has been shown to

deliver intact mRNA molecules to cells yielding production of the corresponding protein.

Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

Other cationic lipid formulations such as formulations incorporating DOTMA have been shown to deliver DNA expression vectors to cells yielding production of the corresponding protein. Lipid formulations may be non-toxic and biodegradable in composition. They display long circulation half-lives and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system. See, Szoka et al.PCT/US93/03406 filed 4/5/93, published 10/14/93.

Other controlled release drug delivery systems, such as nanoparticles and hydrogels may be potential delivery vehicles for a nucleic acid sequence encoding at least one of the therapeutic molecules. These carriers have been developed for chemotherapeutic agents and protein-based pharmaceuticals (such as at least one of the therapeutic molecule proteins), and consequently, can be adapted for nucleic acid delivery. For example formulations can consist of purified DNA vectors or DNA vectors associated with other formulation elements such as lipids, proteins, carbohydrates, synthetic organic compounds, or in-organic compounds. Examples of such formulation elements include, but are not limited to, lipids capable of forming liposomes, cationic lipids, hydrophilic polymers,

polycations (e.g. protamine, polybrine, spermidine, polylysine), peptide or synthetic ligands recognizing receptors on the surface of the target cells, peptide or synthetic ligands capable of inducing endosomal-lysis, peptide or synthetic ligands capable of targeting materials to the nucleus, gels, slow release matrices, soluble or insoluble particles, as well as other formulation elements not listed. This includes formulation element for enhancing the delivery, uptake, stability, and/or expression of genetic material into cells.

A critical step is the delivery of the DNA vector to the nucleus of the target cell where it can express a gene product. In the present invention this may be accomplished by formulation. The delivery and formulation of any selected vector construct will depend on the particular use for the expression vectors. In general, a specific formulation for each vector construct used will focus on vector uptake with regard to the particular targeted tissue, followed by demonstration of efficacy. Uptake studies will include uptake assays to evaluate cellular uptake of the vectors and expression of the tissue specific DNA of choice. Such assays will also determine the localization of the target DNA after uptake, and establishing the requirements for maintenance of steady-state concentrations of expressed protein. Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Elements of a vector delivery system are summarized in the following table.

Complex for gene delivery	
DNA	Condensed plasmid DNA, other DNA could work if super coiled/non-coiled
Complex	sterically stabilized 40-300 nanometer diameter charge, or no charge
Carriers	lipids polymers polyspermine conjugates and other polyamines
Targeting ligands	carbohydrates (tris-galactosyl) carnitine folate bile acids lipoproteins
Endosomal release	GALA-Cys
Nuclear targeting	optional
Formulated product	well-characterized, biodegradable non-immunogenic, low toxicity
Vector for hepatic gene expression	
Promoter/enhancer	albumin, alpha$_1$-antitrypsin, hemopexin, hepatocyte nuclear factor binding elements, serum amyloid A, fibrinogen, prothrombin, apolipoproteins
Controlled persistence	episomal replication, chemical modification

Chemical modification of the nucleic acid sequence encoding at least one of the therapeutic molecules to neutralize negative charge may be all that is required for penetration. However, in the event that charge neutralization is insufficient, the nucleic acid sequence encoding at least one of the therapeutic molecules can be co-formulated with permeability enhancers, such as Azone

or oleic acid, in a liposome. The liposomes can either represent a slow release presentation vehicle in which the modified nucleic acid sequence encoding at least one of the therapeutic molecules and permeability enhancer transfer from the liposome into the targeted cell, or the liposome phospholipids can participate directly with the modified nucleic acid sequence encoding at least one of the therapeutic molecules and permeability enhancer can participate directly with the modified nucleic acid encoding at least one of the therapeutic molecules and permeability enhancer facilitating cellular delivery. In some cases, both the nucleic acid encoding at least one of the therapeutic molecules and permeability enhancer can be formulated into a suppository formulation for slow release.

The nucleic acid sequence encoding at least one of the therapeutic molecules or at least one of the therapeutic molecule proteins may also be systemically administered. Systemic absorption refers to the accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: intravenous, intramuscular, subcutaneous, intraperitoneal, intranasal, intrathecal and ophthalmic. A gene gun may also be utilized. Administration of DNA-coated microprojectiles by a gene gun requires instrumentation but is as simple as direct injection of DNA. A construct bearing the gene of interest is precipitated onto the surface of microscopic metal beads. The microprojectiles are accelerated with a shock wave of expanding helium gas, and penetrate tissues to a depth of several cell layers. This approach permits the delivery of foreign genes to the skin or other tissues of anesthetized animals. This method of administration achieves expression of transgenes at high levels for several days and at detectable levels for several weeks. Each of these administration routes exposes the nucleic acid sequence encoding at least one of the therapeutic

molecules to an accessible targeted tissue. Subcutaneous administration drains into a localized lymph node which proceeds through the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier localizes the nucleic acid sequence encoding at least one of the therapeutic molecules at the lymph node. The nucleic acid sequence encoding at least one of the therapeutic molecules can be modified to diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified nucleic acid sequence encoding at least one of the therapeutic molecules to the cell.

Liposomes injected intravenously show accumulation in the liver, lung and spleen. The composition and size can be adjusted so that this accumulation represents 30% to 40% of the injected dose. The remaining dose circulates in the blood stream for up to 24 hours.

The chosen method of delivery should result in cytoplasmic accumulation and molecules should have some nuclease-resistance for optimal dosing. Nuclear delivery may also be used. Most preferred delivery methods include liposomes (10-400 nm), hydrogels, controlled-release polymers, microinjection or electroporation (for *ex vivo* treatments) and other pharmaceutically applicable vehicles. The dosage will depend upon the disease indication and the route of administration but should be between 1-1000 μg/kg of body weight/day. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon disease delivery vehicle and efficacy data from clinical trials.

Another method of administration involves the use of a DNA transporter system for inserting specific DNA into a cell. The DNA transporter system comprises a plurality of a first DNA binding complex, said complex including a

first binding molecule capable of non-covalently binding to DNA, said first binding molecule covalently linked to a surface ligand, said surface ligand capable of binding to a cell surface receptor; a plurality of a second DNA binding complex, said complex including a second binding molecule capable of non-covalently binding to DNA, said second binding molecule covalently linked to a nuclear ligand, said nuclear ligand capable of recognizing and transporting a transporter system through a nuclear membrane; wherein said plurality of first and second DNA binding complexes are capable of simultaneously, non-covalently binding to a specific DNA.

Additionally, a plurality of a third DNA binding complex may be used, said complex includes a third binding molecule capable of non-covalently binding to DNA, said third binding molecule covalently linked to a virus; wherein said plurality of third DNA binding complexes are capable of simultaneously, non-covalently binding to a specific DNA.

The first binding molecule, the second binding molecule and third binding molecule can each be selected from the group consisting of spermine, spermine derivative, histones, cationic peptides and polylysine. Spermine derivative refers to analogues and derivatives of spermine and include compounds as set forth in International Publication No. WO 93/18759, filed March 19, 1993 and published September 30, 1993 hereby incorporated by reference.

Establishment of therapeutic levels of nucleic acid sequence encoding at least one of the therapeutic molecules within the cell is dependent upon the rates of uptake and degradation. Decreasing the degree of degradation will prolong the intracellular half-life of the at least one of the therapeutic molecules gene. Descriptions of useful systems are provided in the art cited above, all of which is hereby incorporated by reference.

At least one of the therapeutic molecules nucleic acid sequence may be administered utilizing an *ex vivo* approach whereby cells are removed from an animal, transduced with at least one of the therapeutic molecules nucleic acid sequence and reimplanted into the animal. The liver can be accessed by an *ex vivo* approach by removing hepatocytes from an animal, transducing the hepatocytes *in vitro* with at least one of the therapeutic molecules nucleic acid sequence and reimplanting them into the animal (*e.g.*, as described for rabbits by Chowdhury et al, Science 254: 1802-1805, 1991, or in humans by Wilson, Hum. Gene Ther. 3: 179-222, 1992) incorporated herein by reference.

At least one of the therapeutic molecules nucleic acid sequence may be administered utilizing an *in vivo* approach whereby the gene will be administered directly to an animal by intravenous injection, intramuscular injection, or by catheterization and direct delivery of the gene via the blood vessels supplying the target organ. Since at least one of the therapeutic molecules is normally expressed in multiple tissues and organs including heart, skeletal muscle, adipose tissues, spleen, lung, brain, kidney, testis, adrenal, small intestine, and other tissues, any of these tissues can be target organs. Among these tissues, skeletal muscle is one tissue that is readily accessible by intramuscular injection or intravenous injection. Expression will be achieved using a skeletal muscle-specific promoter for the nucleic acid sequence encoding at least one of the therapeutic molecules.

The liver can be accessed by an *in vivo* approach by administration of the nucleic acid sequence encoding at least one of the therapeutic molecules intravenously, intraportally (via the portal vein) or intra-arterially into the hepatic artery.

Many nonviral techniques for the delivery of at least one of the therapeutic molecules nucleic acid sequence into a cell can be used, including direct naked DNA uptake

(*e.g.*, Wolff et al., Science 247: 1465-1468, 1990), receptor-mediated DNA uptake, *e.g.*, using DNA coupled to asialoorosomucoid which is taken up by the asialoglycoprotein receptor in the liver (Wu and Wu, J. Biol. Chem. 262: 4429-4432, 1987; Wu et al., J. Biol. Chem. 266: 14338-14342, 1991), and liposome-mediated delivery (*e.g.*, Kaneda et al., Expt. Cell Res. 173: 56-69, 1987; Kaneda et al., Science 243: 375-378, 1989; Zhu et al., Science 261: 209-211, 1993). Many of these physical methods can be combined with one another and with viral techniques; enhancement of receptor-mediated DNA uptake can be effected, for example, by combining its use with adenovirus (Curiel et al., Proc. Natl. Acad. Sci. USA 88: 8850-8854, 1991; Cristiano et al., Proc. Natl. Acad. Sci. USA 90: 2122-2126, 1993).

The diseases or conditions which are treatable by the methods of this invention and the related protein or polypeptide which may be incorporated into gene therapeutic compositions are as follows:

(1) diabetes mellitus — insulin or proinsulin; (2) cardiovascular disease — apolipoprotein A1, lipoprotein lipase, apolipoprotein E, cholesterol-7α-hydroxylase and combinations thereof; (3) anemia — erythropoietin; (4) growth conditions and aging — growth hormone; (5) Parkinson's disease — tyrosine hydroxylase; (6) hypertension — atrial natriuretic factor; (7) thrombosis — thrombomodulin, protein C, protein S, antithrombin III; (8) hypercholesterolemia — LDL receptor; (9) Metabolic disorders, such as, PKU-PH; and (10) serum disorders, such as, hemophilia A-Factor VIII.

The methods of administration of these compositions and specific examples of compositions and methods for gene therapy for these diseases are set forth below. The following examples are offered by way of illustration and are not intended to limit the invention in any manner.

Example 1: Construction of an Insulin Retrovirus and Expression in Tissue Culture.

The insulin retrovirus LX/rINS, is constructed by cloning the complete sequence for rat preproinsulin 1 gene into a Maloney Murine Leukemia Virus (MMLV) vector developed by Dusty Miller (Miller, A.D., and Rosman, G.J. 1989, BioTechniques 7:980). The insulin gene is under the transcriptional control of the viral Long Terminal Repeat (LTR) promoter (Fig 1A), which is known to be active at constitutive levels for many months in the rat liver (Kolodka, T.M., et al., 1993, Som. Cell Mol. Gen. 19: 491; Ferry, N., et al., 1991, Proc. Natl. Acad. Sci. USA 88: 8377). The control retroviral vector used is LX/hAAT, which bears the human α-1-antitrypsin gene under the transcriptional control of the MMLV-LTR (Kolodka, T.M., et al., 1993, Som. Cell Mol. Gen. 19: 491). The LX/rINS vector is used to transduce rat fibroblast 208F cells and 48 hours later the cultured media are assayed for rat insulin (Fig 1B). Conditioned media from cells which were transduced with buffer alone or with the LX/hAAT had background levels of insulin, and those transduced with the LX/rINS vector contained greater than 600 ng/ml of immunoreactive insulin. Alternatively, a partial length cDNA clone of the rat insulin I gene containing all the coding sequences but missing 33 base pairs at the 5' end was isolated. Synthetic oligonucleotides containing all the missing bases are used as primers for the *in vitro* synthesis of a full length cDNA for rat I insulin which is cloned into the retroviral vector LNCX using a variety of promoters. The promoters include the CMV, PGK, albumin PEPCK, PK and P14 promoters. The recombinant viral DNA construct is introduced into packaging cells to produce virus-producing cell lines with high titers (approximately $1 - 5 \times 10^6$ cfu/ml). This demonstrates that a functional virus is capable of expressing insulin and that a protein normally stored in secretory vesicles can be secreted from

hepatocytes which only have a constitutive secretory pathway.

Example 2: Direct Insulin Gene Transfer *In Vivo*

The procedure developed for *in vivo* retroviral mediated gene transfer into rat hepatocytes has been described in (Kolodka, T.M., et al., 1993, Som. Cell Mol. Gen. 19:491). Briefly, a 70% partial hepatectomy is followed 24 hours later by retroviral infusion into the portal vein. The procedure results in 10-15% hepatocyte transduction *in vivo* and persistent gene expression for at least 6 months.

Hepatocytes of non-diabetic rats are transduced with retroviral vectors, followed by induction of diabetes. 24 hours following a 70% partial hepatectomy, three week old male Lewis rats are infused with LX/rINS; LX/hAAT or media, each with 8 μg/ml polybrene as previously described (Kolodka, T.M., et al., 1993, Som. Cell Mol. Gen. 19:491). Two weeks are allowed to pass after retroviral transduction to allow for liver regeneration. Diabetes is then induced with streptozotocin, a glucose analogue that selectively destroys pancreatic β-cells (Junod, A., et al., 1969, J. Clin. Invest. 48:2129). A very high dose of 250 mg/Kg streptozotocin was chosen to insure complete ablation of pancreatic β-cells and little variability in the severity of diabetes between experimental rats.

Three days after streptozotocin treatment, the control rats are visibly wasted compared to rats treated with the insulin gene. Indeed, in three days, control rats lost approximately 25% of their body weight and the average weight of the group of LX/rINS treated rats remained relatively constant (Fig. 2A). In four days 8/10 LX/hAAT and 8/8 media treated rats succumb, and the 2 remaining rats succumbed by day 6. However, 16/16 of the rats transduced with LX/rINS survived for 19 days, and 13/16 survived 21 days (Fig 2B). Therefore, transduction of rat hepatocytes with the insulin vector appears to have

prevented the early lethal effects of severe diabetes induced by streptozotocin.

Example 3: Prevention of Ketoacidosis in LX/rINS Treated Rats

To determine what specific protective effect the LX/rINS vector was having, sera from all rats were tested for the presence of ketones. Three days after streptozotocin treatment all rats from the two control groups had serum ketone levels of 63±21 mg/dl, which is considered high in diabetic rats. However, all rats treated with LX/rINS had low or no serum ketones (Fig 3A). The slightly elevated ketone levels on day 21 in this group were due to a single rat which had an unusually high ketone level on that day. Thus, the presence of the insulin vector in the hepatocytes of the experimental rats has prevented ketoacidosis associated with severe diabetes.

Serum chemistry analyses were performed to monitor aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) as indicators of hepatic injury, as well as blood urea nitrogen (BUN) and creatinine as indicators of kidney dysfunction (Everett, R.M., and Harrison, S.D. Chapter 13, Clinical Biochemistry In: Foster, H.L., Small, J.D., and Fox, J.G. (Eds.) The Mouse in Biomedical Research Volume III; Normative Biology, Immunology and Husbandry. Academic Press, New York, 1983, 320-323). 14 days after retroviral transduction and prior to streptozotocin treatment, the analyses revealed no differences between the three groups of rats, indicating insulin gene expression from hepatocytes was not harmful to the liver or kidney (Fig. 3B, C). Three days after induction of diabetes however, AST levels increased 8 fold in control rats, but not in rats transduced with the insulin vector (Fig 3B). Increases in LDH levels from 323±189 IU/L to 2929±1929 IU/L and ALT levels from 69±10 IU/L to 218±98 IU/L

suggested acute liver damage in the control rats. In rats transduced with the insulin vector, the levels of ALT, AST and LDH remained in the normal range for the duration of the experiment.

By day 3 after streptozotocin treatment, there was also a 2 fold increase in creatinine levels from 0.6±0.1 mg/dl to 1.2±0.5 mg/dl in the control rats, suggesting renal dysfunction (Fig. 3C). BUN levels also increased in control rats from 19±1 mg/dl to 108±57 mg/dl. In rats transduced with LX/rINS, the levels of BUN and creatinine at 3 days after induction of diabetes were normal (22±8 mg/dl and 0.65±0.1 mg/dl respectively). However, the levels increased gradually afterwards, reaching 79±35 mg/dl for BUN and 1.6±0.6 mg/dl for creatinine at 21 days, suggesting progressive kidney failure. The renal damage was likely a direct effect of streptozotocin as it is a known nephrotoxin at high doses (Schein, P.S. 1972, Cancer 30,1616).

Example 4: Insulin and Glucagon Production in LX/rINS Treated Rats

Rat sera were assayed for the presence of immunoreactive rat insulin via an RIA. 14 days after retroviral transduction and prior to induction of diabetes, non-fasting serum insulin levels in the control groups were 2.2±1.8 ng/ml and those in rats transduced with LX/rINS were 3 fold higher (Fig 4A). The insulin detected in experimental rats was probably a combination of insulin produced by pancreatic β-cells and hepatically produced proinsulin. Three days after induction of diabetes, the levels of serum insulin in control rats decreased 3-6 fold to 0.7±0.7 ng/ml and those in rats treated with LX/rINS increased over 2 fold (Fig. 4A). The reason for the increase is unclear, but it did not appear to be harmful to the rats. Glucagon production by the pancreatic α-cells is also known to increase in poorly controlled type 1 diabetes (Muller, W.A., et al., 1971,

J. Clin. Invest. 50:1992). After retroviral transduction, there was no difference between the three groups of rats with respect to the glucagon levels. Three days after induction of diabetes, the levels of glucagon in the control rats increased 3 to 5 fold as expected. The glucagon levels in rats transduced with the insulin vector remained at levels before the induction of diabetes (Fig. 4B), suggesting that the hepatically produced insulin detected by the radioimmunoassay was active.

Example 5: Histolopathological Examination of the Pancreas, Liver and Kidney

Histological analysis of the pancreas three days after streptozotocin treatment showed islets with misshaped nuclei and highly vacuolated cytoplasm in rats from all three groups. Immunohistological staining of pancreatic sections with anti-rat C-peptide antibodies and anti-glucagon antibodies from rats before the administration of streptozotocin showed very clear staining of α- and β-cells. Three days after treatment with streptozotocin, there was complete ablation of the islet β-cells in all treatment groups. There was no difference in the appearance of the islets from rats transduced with the insulin vector, hAAT or media, suggesting that the insulin gene expression from the liver does not alter the effects of streptozotocin on the β-cells.

Gross histological examination of the liver 14 days after retroviral transduction showed no differences between livers transduced with LX/rINS, LX/hAAT or media. Insulin was not detected by immunohistological staining of the liver, which was expected as the liver does not have the capacity to store secreted proteins. Since insulin is known to promote fat and glycogen storage (Cahill, G.F. 1971, Diabetes 20:785), the distribution of these two macromolecules was determined in livers after retroviral transduction. Before induction of diabetes no fat was detected in any liver section from all three groups of

rats. Glycogen was present in all liver sections and there was no difference in the pattern of glycogen distribution among the three groups of rats. The results suggest that hepatic insulin gene expression does not cause apparent damage to the liver. Three days after the induction of diabetes, large intracellular fat inclusions were evident in all liver sections from the control rats. However, in rats transduced with the insulin vector, only a few small fat inclusions were occasionally detected. There was almost no glycogen detected in livers from control rats, whereas glycogen was apparent in rats transduced with LX/rINS, which was located generally but not exclusively in the periportal regions as in the normal liver. Therefore, the expression of the insulin gene from the hepatocytes appears to have protected the rats from changes in hepatic fat and glycogen contents associated with early acute diabetes. The results corresponded well with those of the serum chemistry analyses.

Example 6: Blood Glucose Levels in LX/rINS Treated Rats

Because the expression of the insulin gene is unregulated and because over expression of insulin is lethal, it was determined if at any time during a typical day the blood glucose levels of the treated rats would drop below the threshold level of 50 mg/dl. Rats are allowed food and water ad libitum and their blood glucose levels are monitored every two hours for periods of 24 hours in order to establish their blood glucose profiles before any experimental manipulation. The blood glucose levels remained fairly constant at approximately 100 mg/dl throughout the 24 hour period. The hepatocytes of these rats are then transduced with either the insulin vector, hAAT of media. 14 days after retroviral transduction, the blood glucose profiles of the rats were determined and again there was no change in the levels of blood glucose between the three groups of rats over the entire 24 hour period. The rats are then treated with streptozotocin at

250 mg/kg and three days later, the blood glucose profiles of all the animals were obtained. As expected, the rats transduced with LX/hAAT and media all had significant increases in blood glucose levels to 250-350 mg/dl and the high levels remained constant for the entire 24 hour period (Fig. 5A). Interestingly, rats transduced with the insulin vector had similarly elevated levels of blood glucose as a group (Fig 5B). The result suggests that the hepatically produced insulin, while sufficient to prevent ketoacidosis in streptozotocin treated animals, was insufficient to restore the treated animals to normoglycemia under non-fasting conditions.

Since the therapy achieved a low level of biologically active insulin production from the liver, test were conducted to see if these levels of insulin activity were sufficient to lower blood glucose levels in the rats under fasting conditions. A 24 hour fasting blood glucose profile was thus obtained. The blood glucose levels in control rats remained at non-fasting levels (250-350 mg/dl) for the first 10 hours, then slowly decreased over the next 14 hours (Fig. 6A). During this 14 hour period, the rats became lethargic, and unconscious, and 3 of the rats died due to severe diabetes. The blood glucose levels in the LX/rINS treated group decreased to normoglycemic levels (90-110 mg/dl) within the first 4 hours of the fast, and remained in this range for 20 hours (Fig. 5B). At no point during the first 18 hours did the blood glucose level of any of the LX/rINS treated rats decreased to levels below 50 mg/dl. Therefore, sufficient insulin activity was produced in the liver to modify blood glucose levels in these rats under fasting conditions.

Example 7: Glucose-Responsive Promoter

A glucose regulated promoter for insulin gene expression can also be used in the above described vector. However, even without such a glucose regulated promoter, it has been demonstrated unambiguously that a

constitutive, fasting level of insulin activity produced from the hepatocytes of severely diabetic rats can prevent the acute lethal consequences of insulin dependent diabetes mellitus. A regulatable promoter is desirable to control the level of insulin production *in vivo* in humans. The preferred insulin regulated promoter is the phosphoenolpyruvate carboxykinase (PEPCK) gene. It is a hepatic promoter which has been extensively studied and well characterized. It is transcriptionally activated by cAMP, glucocorticoids and glucagon, and is inhibited by insulin. Inhibition by insulin is of great importance since this prevents the potential condition of hyperinsulinanemia which is untreatable and lethal. Further, other glucose responsive promoters such as pyruvic kinase and P14 also can be used to regulate ectopic insulin production in the liver.

Example 8: Proinsulin to Insulin Conversion

Mature insulin consists of two polypeptide chains, A and B connected by disulfide bonds. A precursor to insulin, proinsulin, is a single peptide in which the A and B peptide genes are connected together by the C polypeptide. Proinsulin molecules have 2% to 5% of the activity of the mature insulin molecule. Normally the C peptide is excised in the secretory vessels of the pancreatic β-cells by two endopeptidases.

In the present invention the insulin gene is inserted into the hepatocytes which do not contain the two endopeptidases. Thus cleavage must rely on liver endopeptidases. The propeptides synthesized in the liver are cleaved by hepatic endopeptidase at the consensus sequence Arg-X-Lys-Arg or Arg-X-Arg-Arg. X may be any amino acid. The B chain/C peptide junction of human proinsulin conforms to the Arg-X-Lys-Arg sequence (residue 31 to 34). The A chain/C peptide junction, however, does not conform (residue of 62-65). When human proinsulin is added to hepatic vessels, the proinsulin is cleaved at the

B/C junction but not at the A/C junction. These partially cleaved molecules have the same biological activities as unprocessed proinsulin. Hepatocytes, like other cell types with a constitutive secretory pathway, process secretory proteins by the enzyme furin, which cleaves at specific amino acid sequences (Hosaka, M., et al., 1991, J. Biol. Chem. 266:12127). The sequence at the A/C junction, but not at the B/C junction of the rat insulin 1 protein, conforms to the furin recognition sequence, which presumably results in the incomplete cleavage of proinsulin. Recently Groskreutz et al. have demonstrated that if the A/C and B/C junctions of human proinsulin gene are converted to the furin recognition sequence, the proinsulin is fully processed in a human kidney cell line via the constitutive secretory pathway (Groskreutz, D.J., et al., 1994, J. Biol. Chem. 269: 6241). Expression of these mutant insulin genes in the liver of diabetic animals can result in the production of mature insulin. Because of this full biological activity, this will allow the reduction of viral dosages for treatment of insulin-dependent diabetes.

In order to overcome this problem the gene for proinsulin can be modified by site directed mutagenesis. The amino acid sequence at the A/C junction of human proinsulin (amino acid residue of 62-65) are changed to that of the consensus sequence known to be cleaved by hepatic endopeptidases. The new sequence at the A/C junction can then be processed by the type I convertase that is present in hepatocytes, as well as other non-pancreatic beta cells. The mutation results in the production of normal sequences for both the A and B chains of human insulin since the amino acid substitutions are only made in the C peptide, which is not present in the mature insulin molecule. Thus, this allows for the production of mature insulin from ectopic tissues for the treatment of diabetes.

Example 9: Proinsulin vs. Processed Insulin

Another alternative strategy that is employed to generate insulin in the hepatocyte is to co-infect hepatocytes with the A-chain cDNA and the B-chain cDNA. This can be done in a single vector or with multiple vectors. By adding appropriate cellular translocation signals, the cDNA for each insulin chain can be co-expressed in the hepatocytes. A certain percentage of the A and B chain will correctly associate to form intact insulin molecules prior to secretion. This however is less efficient than Example 8.

Example 10: Treatment of Parkinson's Disease

A retroviral construct containing the tyrosine hydroxylase ("TH") nucleic acid sequence is shown in Figure 11. In this construct the TH cDNA is driven by the liver-specific albumin promoter. One skilled in the art will recognize that many other promoters can also be used, depending on the type of target cells and desired level of L-Dopa expression. The retrovirally transduced hepatocytes are reintroduced into the liver by direct injection into the portal vein of the spleen. The implanted cells migrate to the liver, embed themselves in the liver parenchyma and function as hepatocytes for the life of the recipient animal.

A number or viral and non-viral methods can be used to deliver the tyrosine hydroxylase gene to cells, such as hepatocytes. Viral-based methods include adenovirus, adeno-associated virus, vaccine virus, herpes simplex virus and others. Non-viral delivery methods include naked DNA, liposome-DNA complexes, protein-DNA complexes, chemical-DNA complexes and others. For viral-based methods, transduction could occur either *in vivo* or *in vitro*. For non-viral methods, the tyrosine hydroxylase nucleic acid sequence is introduced directly into the target cell *in situ*.

Example 11: Treatment of Cardiovascular Disease

In order to treat cardiovascular diseases, it is best to achieve high serum concentrations of HDL (High Density Lipoproteins) and low levels of LDL (Low Density Lipoproteins). This can be accomplished by over expressing a combination of four proteins in the liver. The proteins are apoA-1, apoE-4, LPL that has been modified to remove its heparin binding site and cholesterol-7α-hydroxylase.

Recombinant retrovirus containing the genes for human apoA-1, human apoA-4, human LPL, human cholesterol-7α-hydroxylase or combination thereof can be constructed. A full-length cDNA clone of human apoA-1 containing all of the coding sequences is incorporated into a retroviral vector LNCX utilizing a variety of promoters by the method described above. The same strategy is used to incorporate the full-length human apoA-4, LPL, and cholesterol-7α-hydroxylase into a retroviral vector. In some cases, more than one gene and possibly all four genes can be incorporated into a viral vector. For example, apoA-1 and cholesterol-7α-hydroxylase. The recombinant viral DNA construct is introduced into the packaging cells to produce virus producing cell lines with high titers (1-5 X 10^6 cfu/ml). The apoA-1 virus is used to infect two human hepatoma cell lines, Hep G2 and PLC. After 36-48 hours post-viral infection, a sensitive radioimmunoassay is used to determine the levels of apoA-1 secretion.

Because of its ease in producing hypercholesterolemia by cholesterol feeding, the C57BL/6 mouse is a good example for showing the effect of these vectors. Hepatocytes from this inbred strain are isolated and infected with apoA-1 virus or the DNA vector under optimal infection conditions. The hepatocytes are then transplanted into mice via splenic injection. Because the donor cells in the recipients have identical genetic backgrounds, there is no immunological rejection of the graft by the host. The efficacy of this therapeutic

approach is monitored by a simple observation of serum cholesterol and triglyceride levels, lipoprotein profiles and apoprotein levels. After intravenous injection in experimental animals, (Huang, S-W, and Maclaren, N.K., 1976 Science 192:64) the tissue localization of the DNA, (2) the tissue specificity for gene expression, (3) how long the new gene can be expressed and (4) function is determined.

In treating patients, a regulator promoter is desirable in order to control the level of apoA-1 production *in vivo*. There are several positive and negative promoter regions identified for the human apoA-1 gene.

A number or viral and non-viral methods can be used to deliver the at least one of an apoA-1, human apoA-4, human LPL, human cholesterol-7α-hydroxylase or combination thereof gene to cells, such as hepatocytes. Viral-based methods include adenovirus, adeno-associated virus, vaccine virus, herpes simplex virus and others. Non-viral delivery methods include naked DNA, liposome-DNA complexes, protein-DNA complexes, chemical-DNA complexes and others. For viral-based methods, transduction could occur either *in vivo* or *in vitro*. For non-viral methods, the above-listed protein nucleic acid sequence is introduced directly into the target cell *in situ*.

Example 12: Treatment of Hypercholesterolemia

English Half-lop rabbits bearing the Watanabe Heritable Hyperlipidemic Rabbit trait (EHL-WHHR) were bred at The Rogosin Institute at the New York Hospital-Cornell Medical Center. Ten females were superovulated and artificially inseminated to produce enlarged litters of the same age for these experiments. Litter mates (polyzygotic) were then sex-matched across experimental groups to minimize variability, thereby increasing the statistical power of the experimental design (Donnelly, T. M., et al., 1991. *J. Lipid Res.* 32:1089-98).

Construction of Recombinant Adenovirus with Rabbit LDL Receptor Gene

Approximately 200 bp of DNA is added to the 5' end of the rabbit LDL receptor cDNA clone in order to regenerate the translation initiation codon and the signal peptide. This is done by annealing and ligation of overlapping oligonucleotides designed according to the human LDL receptor sequence (Sudhof, T. C., et al., 1985. *Science* 228:815-22). The resultant cDNA fragment is cloned into the EcoR V site of the E1A- adenovirus transfer vector pAdL1/RSV (Graham F. L., L. Prevec. 1991. Manipulation of adenovirus vector, *In* Methods in molecular biology: gene transfer and expression protocols, Vol.7. Murray, E. J., editor. The human press. Clifton, NJ, 109-128; Brantly, M., et al., 1988. *Science* 242:1700-2). This construct is cotransfected with the plasmid pJM17 into the adenovirus packaging cell line 293. Recombinant adenovirus clones were plaque purified and amplified as previously described (Kay, M. A., et al., 1994. *Proc. Natl. Acad. Sci. USA* (in press)). The viral titer, as determined by OD, varied from 1 to 3 x 10^{11} particles/ml.

Rabbit LDL receptor functional assay

In vitro binding, uptake and degradation of [^{125}I]-labelled LDL by primary hepatocytes of Watanabe rabbits was measured after transduction by Adv/RSV-rbLDLR *in vitro* to confirm the function of the rbLDLR cDNA. Watanabe rabbit hepatocytes were isolated and cultured in SUM medium (tyrosine-free MEM/Waymouth's supplemented with growth factors) overnight (Cristiano, R. J., et al., 1993. *Proc. Natl. Acad. Sci. USA* 90:2122-6). The hepatocytes were transduced with Adv/RSV-rbLDLR at 0, 1, 10 and 100 particles/cell for 4 hours. The medium was then changed and the cells were cultured overnight. Binding, uptake and degradation of [^{125}I]-labelled LDL were measured as described (Goldstein, J. L., et al., 1983. *Meth. Enzymol.* 98:241-260; Mims, M. P., et al., 1990. *Biochem.* 29:6639-47).

Example 13: *In vivo* Delivery of Recombinant Adenovirus Via Splenic Vein Infusion

New Zealand White ("NZW") rabbits were anesthetized with 0.5 ml/kg of an anesthetic containing a combination of ketamine (1.43 mg/ml), xylazine (0.286 mg/ml) and acepromazine (0.047 mg/ml). EHL-WW rabbits were anesthetized using the respiratory anesthetic Aerrane (isoflurane). Recombinant adenovirus suspended in 10 ml of buffer was then infused through splenic vein injection.

To test LDL receptor gene expression *in vivo*, the adenovirus Adv/RSV-rbLDLR was infused into the portal circulation of Watanabe rabbits. Hepatocytes were isolated from these rabbits three days post infusion, and the binding and uptake of [^{125}I]-labelled LDL were measured as described above. Hepatocytes from rabbits infused with a recombinant adenovirus bearing the human α1-antitrypsin gene (Adv/RSV-hAAT) were used as a negative control.

Example 14: Virus Transfection Efficiency and Tissue Distribution

Adv/RSV-ßgal particles at 0, $1x10^{11}$, $2x10^{11}$, $4x10^{11}$ particles/kg body weight were infused into NZW rabbits. Three days after infusion, hepatocytes are isolated and stained for ß-galactosidase activity as previously described (Li, Q. T., et al., 1993. *Hum. Gene Ther.* 4:403-9) to determine the *in vivo* transduction efficiency. A sample of liver tissue is also collected at this time for histopathological analysis. At one week or four weeks post-infusion, ten different tissue types were collected. Genomic DNA was extracted from frozen tissue samples, and serial dilutions containing 1 μg to 1 ng were used as templates in a semi-quantitative PCR assay to determine the presence of Adv/RSV-ßgal DNA as previously described (Kay, M. A., et al., 1994. *Proc. Natl. Acad. Sci. USA* (in press)). DNA isolated from 1 to 10^6 viral particles was amplified in parallel as positive controls. A 500-bp PCR product was generated from amplification with primers

located in the RSV promoter (5'-GTAAGGTGGTACCATCGT-3') and the ß-galactosidase gene (5'-GGATGTGCTGCAAGGCGA-3'). Viral DNA copy numbers per cell were determined by comparing the band density of these products on agarose gel to those of PCR products from the positive controls.

Example 15: Plasma Total, HDL- and LDL-Cholesterol, and Apolipoprotein AI Determinations

Six sets of age- and sex-matched Watanabe rabbits were infused with recombinant Adv/RSV-rbLDLR, Adv/RSV-hAAT or buffer through splenic vein injection. Plasma cholesterol level was determined twice weekly both pre- and post-infusion in all animals. Rabbits infused with Adv/RSV-hAAT and buffer served as negative controls. Total plasma cholesterol level was determined by an enzymatic method using the Cholesterol/HP kit from Boehringer Mannheim Diagnostics in a Hitachi 704 Analyzer. HDL-cholesterol level was measured from the supernatant after precipitating the other plasma lipoproteins with 0.05 M $MgCl_2$ and 0.05% dextran sulfate (Burstein, M., et al., 1970. *J. Lipid Res.* 11:583-95). An enzyme-linked immunosorbent assay (ELISA) with a goat anti-rabbit ApoAI IgG from Parke-Davis was used to determine serum Apo AI levels (Lin, R.C. 1986. *Anal. Bioch.* 154:316-26).

Example 16: Functional Assay of the recombinant Adv/RSV-rbLDLR Vector

A recombinant adenovirus containing the rabbit low density lipoprotein receptor ("rbLDLR") cDNA under the transcriptional control of the Rous sarcoma virus ("RSV") long terminal repeat ("LTR") promotor was constructed ("Adv/RSV-rbLDLR"). The functionality of the recombinant vector was assayed by measuring the binding, uptake and degradation of [^{125}I]-labelled LDL in LDL receptor-deficient Watanabe rabbit hepatocytes after viral transduction *in vitro* (Fig. 6). While non-transduced Watanabe hepatocytes exhibited minimal uptake and degradation of ^{125}I-LDL, the

Adv/RSV-rbLDLR vector successfully restored LDL receptor function in the LDL receptor-deficient hepatocytes in a dose-dependent manner.

In a pilot experiment, two Watanabe rabbits were infused with $4x10^{11}$ particles/kg of Adv/RSV-rbLDLR. Three days after infusion, hepatocytes isolated from rabbits receiving the rbLDL receptor gene bound and internalized six times more [^{125}I]-LDL than hepatocytes isolated from animals treated with the control virus Adv/RSV-hAAT (193.25 ± 17.99 ng/mg cell protein vs. 32.25 ± 4.10 ng/mg cell protein).

Example 17: Hepatocyte Transduction Efficiency and Tissue Distribution of viral DNA after Splenic Vein Infusion of Adv/RSV-ßgal in Normal Rabbits

The efficiency of adenovirus-mediated gene delivery to rabbit hepatocytes *in vivo* was determined by infusing various doses of the recombinant adenovirus Adv/RSV-ßgal into the splenic vein of New Zealand White rabbits. Buffer infusion served as a negative control. The hepatocytes were isolated two days post injection, followed by staining with X-gal. 80% and 88% of hepatocytes were stained blue at doses of $4x10^{11}$ and $10x10^{11}$ viral particles/kg body weight respectively. Semi-quantitative PCR assay was used to estimate the amount of recombinant adenoviral DNA in brain, kidney, heart, liver, lung, muscle, pancreas, spleen, stomach, and testes after *in vivo* infusion of $4x10^{11}$ viral particles/kg. One week after viral infusion, viral DNA concentrations were detected in the liver and spleen. The concentration were lower by 1-2 orders of magnitude in the kidney, lung, stomach and pancreas. Less than 1 copy per 1,000 cells were detectable in the brain, heart and skeletal muscle. Four weeks after infusion, viral DNA sequences became negligible in all tissues.

Example 18: Plasma Cholesterol Reduction in Watanabe Rabbits After Delivery of the rbLDLR Gene

Eighteen Watanabe rabbits were divided into six age- and sex-matched sets. One member of each set was infused with either $4x10^{11}$ particles/kg of Adv/RSV-rbLDLR, Adv/RSV-hAAT, or buffer through splenic vein injection. Six days after treatment, plasma total cholesterol in the animals treated with Adv/RSV-rbLDLR was significantly decreased relative to their pretreatment levels (825.5 ± 69.8 mg/dl vs. 247.3 ± 61.5 mg/dl; $p < 0.001$) (Fig. 8). These levels were also significantly lower than those observed in either of the two control groups ($p < 0.001$), despite a small but significant decrease in plasma total cholesterol levels in the two groups over this same time period (819.0 ± 121.9 mg/dl vs. 667.2 ± 96.1 mg/dl; p vs. 0.05). Plasma total cholesterol values in the animals receiving Adv/RSV-rbLDLR reached their lowest levels one week after infusion and then gradually increased to control levels after three weeks. Thus, this recombinant vector efficiently reduced plasma cholesterol in the Watanabe rabbits.

Example 19: Elevation of Plasma HDL-Cholesterol and Apolipoprotein AI in Watanabe Rabbits after Delivery of the rbLDLR Gene

Treatment with Adv/RSV-rbLDLR also caused a significant elevation in HDL-cholesterol and Apo AI levels. Ten days after treatment, HDL-cholesterol levels in these animals were increased by 4-5 fold from relative to their own pretreatment levels (19.8 ± 4.0 mg/dl vs. 4.0 ± 0.0 mg/dl; $p < 0.005$) and to those observed in either of the two control groups ($p < 0.01$) (Fig. 8). At this same time point, Apo AI levels in animals treated with Adv/RSV-rbLDLR (6.44 ± 0.34) were also significantly increased relative to those infused with either Adv/RSV-hAAT (0.97 ± 0.76; $p < 0.001$) or buffer (1.54 ± 1.11; $p < 0.005$) (Fig. 9). HDL-cholesterol and Apo AI both returned

to the pretreatment range by three weeks. These effects were specific for the rbLDLR gene, since it was not observed in buffer- or control virus-treated groups. A most intriguing pattern emerged when the time course of the plasma total cholesterol and HDL-cholesterol responses are compared (Fig. 10). While the lowest plasma cholesterol levels were reached in the Watanabe rabbits six days after delivery of the rbLDLR gene, the highest plasma HDL-cholesterol levels were reached at 10 days post-treatment.

Example 20: Treatment of Hypertension By Hepatic Gene Therapy

ANF can be expressed in the liver or other ectopic (non-heart sites) by the methods described below. ANF is a peptide hormone actively secreted by the heart, released in response to atrial distention, with multiple target organ effectors. It increases sodium excretion, inhibits the renin-angiotensin-aldosterone (RAA) system, and decreases arterial pressure. As such, ANF represents the first clearly documented cardiac hormone (Cody, R.J., 1990, Ann. Rev. Med. 41:377-382). The precursor peptide of ANF (pro-ANF) is a 126-amino-acid residue peptide that is stored in atrial myocytes of mammalian tissue. Several structurally related ANF segments have been isolated; the characteristics of the peptide are summarized by Weber et al. (Weber et al., 1987, Hypertension 10:582-589). The circulating form of ANF in humans corresponds to residue numbers 99 to 126, which comprise the C-terminal 28-residue peptide of pro-ANF.

ANF levels in the blood of normal subjects increases with small increases in dietary sodium intake (Sagnaella, G.A., et al., 1990, Am. J. Hypertens. 3:863-5), and ANF is probably involved in the control of vascular volume. ANF may serve to protect the central circulation from volume and pressure overload (Burnett, J.C. Jr., 1990, Circulation 82:1523-4). This action is mediated by

fundamental actions on cardiovascular, renal, and endocrine systems. Such homeostatic actions appear to include preventing excessive tachycardia via modulation of baroreceptors, limiting activation of the RAA system as well as maintaining sodium excretion despite the stimulus of arterial hypotension. Commercially prepared ANF is degraded by gastrointestinal proteases and therefore must be administered intravenously.

In addition to hypertension chronic congestive heart failure ("CHF") appears to be amenable to treatment with hepatic gene therapy encoding the ANF gene. Cody and colleagues (Cody, R.J., et al., 1986, J. Clin. Invest. 78:1362-74) reported the action of the peptide ANF administration on integrated cardiovascular-renal-endocrine function in humans with CHF. ANF administration in normal subjects produces a brisk natriuresis and diuresis, inhibition of the RAA system, and a reduction in pulmonary capillary wedge pressure. In chronic CHF, ANF gives a striking increase in GFR and renal blood flow in association with inhibition of plasma renin activity.

An alternative gene that may be expressed in the liver for the treatment of hypertension is γ-melanocyte stimulating hormone (γ-MSH). Pro-opiomelanocortin is a large precursor protein found in pituitary corticotrophs and in other brain regions (Humphreys, M.H., Lin, S.-Y., 1988, Hypertension 11:397-410). It gives rise to a number of peptides in the peripheral circulation, including adrenocorticotropic hormone (ACTH), β-endorphin and α-melanocyte stimulating hormone, β-MSH and γ-MSH; the initial N-terminal sequence bears structural similarity to calcitonin. Infusion of low doses of γ-MSH has been shown to lead to natriuresis (Humphreys, M.H., Lin, S.-Y., 1988, Hypertension 11:397-410).

The full length cDNA coding sequences of either rat or human ANF or γ-MSH are incorporated into the retroviral vector LNCX as described above. Liver-specific or tissue-nonspecific promoters are recombined with the coding

sequence of the gene in the proper orientation and the DNA construct is introduced into the packaging cell lines as described above to produce high titers of recombinant virus (1-5x10^6 cfu/ml). The spontaneously hypertensive rat has been described as an ideal animal model for the testing of various therapies because of the ease and reproducibility of producing hypertension by dietary NaCl loading (Hilbert, P., et al., date, Nature 353:521-9; Pollack, D.M., Arendshort, W.J., 1990, Hypertension 16:72-9; Jin, H., et al., 1988, Hypertension 11:738-44). Hepatocytes from this inbred strain are isolated and infected with either the human ANF virus or the vector virus under optimal infection conditions (see above). The hepatocytes are then transplanted into the rat via splenic injection. The efficacy of the treatment is monitored by measuring the level of circulating serum human ANF and the reduction of hypertension. After intravenous injection in experimental animals, a) the tissue localization of the DNA, b) the tissue specificity for gene expression and c) how long the new gene can be expressed and function is determined. For future therapy in patients, a regulatable promoter as described above may be used to control the level of ANF production.

Direct injection of the retroviral vectors can also be performed. In addition to retroviruses alternative delivery systems can be employed. Adenovirus, adeno-associated virus, vaccinia virus or herpes virus vectors can be used to introduce the ANF gene in *ex vivo* treatment of *in vivo* administration. Non-viral vectors can also be employed including liposome-DNA, protein-DNA or chemical-DNA formulations. The ability to express a given gene in ectopic tissues to treat hypertension is not limited to the liver. This approach can be readily modified to provide for gene expression in all other cell types by the appropriate selection of active control elements and delivery systems.

Example 21: Hepatic Gene Therapy for Treatment of Anemia

Deliver of erythropoietin cDNA to the liver of individuals who suffer from anemia secondary to irreversible renal damage or due to chronic illness that results in anemia is achieved as follows. The result of this therapy will be correction of the anemia. For treatment of anemia, a portion of the liver is removed from a patient with immediate preparation and culture of the hepatocytes *in vitro*. The cells are then transduced with a retroviral vector as described below that is designed to produce erythropoietin. The cells are transplanted back into the portal circulation where they take residence in the liver indefinitely. Serum EPO is monitored by RIA or ELISA with a human specific antibody before and after treatment. The blood hematocrit and hemoglobin are monitored as known in the art.

A retroviral vector is constructed (figure 11) that contains the human erythropoietin cDNA under the transcriptional control of the albumin promoter-enhancer (Pinkert, C.A., et al, 1987, Genes and Development 1: 268-76). The construct is transfected into an amphotropic packaging cell line and high titer colonies will be isolated by routine methods. Amphotropic retroviral vectors infect hepatocytes with a relatively high transduction efficiency (Kay, M.A., et al., 1992, Proc. Natl. Acad. Sci., U.S.A. 89:89). The transduction efficiency is monitored by DNA blots from samples obtained from infected hepatocytes. Additionally, the erythropoietin production is measured *in vitro* by measuring the amount of this specific protein secreted into the media. The albumin promoter is initially used because it represents a strong liver-specific promoter however, other promoters may be substituted as necessary.

The use of erythropoietin production in the liver can also be achieved by other methods. These methods include the use of other viral or non-viral vectors for *in vitro* therapy as well as direct *in vivo* delivery to hepatocytes.

These include adenovirus, adeno-associated virus, vaccinia virus, herpes virus, formulated DNA complexes, protein-DNA complexes, and chemical-DNA complexes. The gene transfer technologies described herein are also applied to other organs and tissues to achieve erythropoietin production and secretion into the circulation to achieve the same medical benefits.

Example 22: Gene Therapy for Thrombosis

Hemostasis involves the interaction of a number of plasma proteins and platelets at the endothelial cell surface of blood vessels (Hedner, U., Davie, E.W. 1989 Introduction to Hemostasis and the Vitamin K-dependent Coagulation Factors, in *Metabolic Basis of Inherited Disease*, Sixth Edition (Scriver CR, Beaudet AL, Sly WS, Valle, D Eds) McGraw-HilL NY, NY, pp 2107-21341). Platelets aggregate at the site of vascular injury. A fibrin clot is formed by the blood coagulation cascade. At least four mechanisms aid in preventing vascular occlusion by platelet plugs or fibrin deposition. These mechanisms include the fibrinolytic pathway, the enhancement of action of endogenous inhibitors of coagulation, the inhibition of cofactors required for fibrin formation, and the synthesis and release of platelet inhibitors.

The endothelial cell surface contains a thrombin binding protein, thrombomodulin, which, when complexed with thrombin, rapidly activates vitamin K-dependent protein C, an endogenous anticoagulant. Protein C and its cofactor, protein S, inhibit the clotting process by inactivating factors Va and VIIIa and, additionally, enhance the lysis of fibrin. It has been demonstrated by the detection of heterozygous and homozygous protein C-deficient patients with severe thrombotic complications that protein C is a major regulatory protein of hemostasis and thrombosis. In the presence of protein C, endocytosis of thrombin:thrombomodulin complexes is inhibited.

Activated protein C does not inhibit endocytosis of thrombin: thrombomodulin complexes. Clearance of thrombin on the vascular endothelium *in vivo* also occurs at heparin-like binding sites which serve as cofactors for the thrombin-antithrombin III reaction. Thrombin clearance, which occurs following reaction with antithrombin III or thrombomodulin, probably takes place at different stages in hemostasis.

Over expression of genes for thrombomodulin, protein C, protein S, and antithrombin-III, either alone or in combination, in endothelial cells and/or in the liver greatly enhances the antithromogenic properties of the vascular endothelium. The activated partial thromboplastin time (APTT and thrombin clotting time (TCT) were both prolonged, whereas there was no effect on the prothrombin time. Although protein C, protein S, and antithrombin-III are normally expressed the liver, they have also been expressed in other cell lines (Chang G.T., et al., 1992 Thromb. Haemost. 67:526-32; Nelson R.M., 1991, J. Biol. Chem. 266:20586-9; Gillespie, L.S., et al., 1991, J. Biol. Chem. 266:3995-4001). The recombinant protein C and protein S are active by coagulation criteria, provided there is sufficient vitamin K in the culture media (Chang G.T., et al., 1992 Thromb. Haemost. 67:526-32; Nelson R.M., 1991, J. Biol. Chem. 266:20586-9).

DNA complexes have been designed for high efficiency direct delivery of these genes to endothelial cells and/or hepatocytes *in vivo*. The DNA complexes have at least three components: 1) the DNA containing the genes of interest and appropriate promoter, enhancer and other cis-acting elements 2) poly-L-lysine and/or other polycations to condense the DNA to a torus structure, small enough to undergo endocytosis; and 3) a ligand for endothelial and/or hepatocyte cell surface receptors. The DNA complex may also include a nuclear localization sequence attached

to poly-L-lysine and a fusion competent, replication-defective adenovirus attached to poly-L-lysine.

Examples of the cell ligands and their respective receptors are (a) thrombin and thrombomodulin; (b) hepatocyte growth factor; (c) transferrin; (d) interleukin 1; (e) endothelial cell growth factor; (f) mannose-6-phosphate and mannose-6-phosphate/insulin like growth factor-II receptor; (g) atrial natriuretic factor (pulmonary endothelial cells); (h) tumor necrosis factor; and (i) mannose (liver endothelial cells). In general, recombinant peptide ligands are modified by site specific mutagenesis, so that these ligands (a) can be covalently linked to polycations or to DNA and (b) retain high affinity receptor binding while undesirable biological effects are abolished. Mutagenesis studies have identified the unique functional regions of many of these ligands. The hepatocyte cell ligands and their respective receptors are (a) L-aspartoyl-bis-α,β-[6-aminohexanamido-tris-(β-lactopyranosyl-hydroxymethyl)-aminomethane], [asp(tris-lacAHT)$_2$] and the asialoglycoprotein receptor, (b) folic acid and the folate receptor and (c) hepatocyte growth factor, which are functional *in vivo*.

Examples of endothelial specific promoters include (a) endothelin-1 promoter; (b) tissue plasminogen activator type 1 promoter; and (c) vascular cell adhesion molecule-1 promoter. In addition to the liver specific albumin promoter, promoters from various viral and cellular genes include long terminal repeat (LTR); cytomegalovirus (CMV) immediate early; Rous sarcoma virus (RSV); and phosphoglycerol kinase (PGK).

The anticoagulant effects resulting from expression of genes for thrombomodulin, protein C, protein S, and antithrombin-III, either alone or in combination, in vascular endothelium and/or the liver or other cells should greatly reduce morbidity and mortality from stroke, coronary artery disease, peripheral vascular disease and pulmonary embolism.

Example 23: Hepatic Gene Therapy with Growth Hormone Vectors

Gene therapy formulations containing a growth hormone cDNA, *e.g.*, human, can be delivered to the liver of individuals who suffer from growth delay secondary to a variety of reasons. Moreover, since growth hormone therapy can slow the aging process then the use of this type of therapy has wide spread applications. The result of this therapy are the correction of the growth deficit and/or inhibition of the aging process. The procedure involves removing a portion of the liver from a patient with immediate preparation and culture of the hepatocytes *in vitro*. The cells are transduced with a retroviral vector as described below that is designed to produce growth hormone. The cells are then transplanted back into the portal circulation where they will take residence in the liver indefinitely. Serum growth hormone is monitored by RIA or ELISA with a human specific GH antibody before and after treatment. The blood somatemedins may also be monitored in a similar manner.

A retroviral vector constructed as shown in figure 11 contains the human growth hormone cDNA under the transcriptional control of the albumin promoter-enhancer (Pinkert, C.A., et al., 1987, Gene and Devel. 1:268-76). The construct is transfected into an amphotropic packaging cell line and high titer colonies are isolated by methods as described above. Amphotropic retroviral vectors infect hepatocytes with a relatively high transduction efficiency. The transduction efficiency are monitored by DNA blots from samples obtained from infected hepatocytes. Additionally, the growth hormone production is measured *in vitro* by measuring the amount of this specific protein secreted into the media. The albumin promoter is used in this present construction because it represents a strong liver-specific promoter however, other promoters may be substituted as necessary.

The production of growth hormone gene therapy in the liver can also be carried out by other methods. These methods involve other viral or non viral vectors for *in vitro* as well as direct *in vivo* delivery to hepatocytes. These include adenovirus, adeno-associated virus, vaccinia virus, herpes virus, liposome-DNA complexes, protein cDNA complexes, and chemical-DNA complexes. Finally, the ability to express the growth hormone gene to treat dwarfism, Turner's Syndrome as well as aging is not limited to the liver. With the judicious choice of tissue specific promoters, the gene can be targeted to a variety of organs and tissues to achieve growth hormone production and secretion into blood to achieve the medical benefits.

The growth hormone gene therapy technology described is also applied in the agricultural industry. It is well known that growth hormone administration in cattle raises milk production and may increase their growth rate of farm animals. Administration of the peptide hormone is effective but very labor intensive and the protein is expensive to produce and purify. Another alternative is to create transgenic farm animals to express the growth hormone gene in ectopic tissues. In addition, the *in vivo* delivery of growth hormone gene into somatic tissues of farm animals can be done by a single DNA infusion and the effect will last the life of the animals.

Example 24: Hepatic Gene Therapy for Metabolic Disorders

Gene therapy formulations containing cDNA for selected enzymes can be constructed to be delivered to the liver or other target organs of individuals suffering from genetically inherited metabolic disorders. It is known in the art how to identify the particular enzyme that is deficient in many common inherited genetic disorders of metabolism. Once identified the appropriate expression vector can be constructed by the methods described above. The more common enzymes deficiencies that are found to be the cause of inherited genetic disorders of metabolism and

can be introduced into patients includes phenylalanine hydroxylase ("PAH") the cause of PKU, methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase. The result of this therapy is the correction of the enzyme deficiency and minimization or prevention of the detrimental effects of these metabolic disorders. in one aspect, the procedure involves removing a portion of the liver from a patient with immediate preparation and culture of the hepatocytes *in vitro*. The cells are transduced with a retroviral vector as described below that contains for example the cDNA for PAH. The cells are then transplanted back into the portal circulation where they will take residence in the liver or other target organ indefinitely. Serum PAH levels are monitored by RIA or ELISA with specific antibody before and after treatment.

A retroviral vector constructed similar to the ones shown in figure 11 is constructed containing PAH cDNA under the transcriptional control of the albumin promoter-enhancer (Pinkert, C.A., et al., 1987, Gene and Devel. 1:268-76) or other suitable promoters. The construct is transfected into an amphotropic packaging cell line and high titer colonies are isolated by the methods described above. Amphotropic retroviral vectors infect hepatocytes with a relatively high transduction efficiency. The transduction efficiency are monitored by DNA blots from samples obtained from infected hepatocytes. Additionally, PAH production is measured *in vitro* by measuring the amount of this specific protein secreted into the media. The albumin promoter is used in this present construction because it represents a strong liver-specific promoter however, other promoters may be substituted as necessary.

The introduction of the expression vector containing PAH cDNA can also be carried out by other methods. These methods involve other viral or non-viral vectors for *in vitro* as well as direct *in vivo* delivery to hepatocytes.

These include adenovirus, adeno-associated virus, vaccinia virus, herpes virus, liposome-DNA complexes, protein cDNA complexes, and chemical-DNA complexes.

Example 25: Hepatic Gene Therapy for Serum Protein Disorders

Gene therapy formulations containing serum protein cDNA can be delivered to the liver of individuals who suffer from serum protein deficiencies such as hemophilia. The result of this therapy are the correction of the disease. in one aspect, the procedure involves removing a portion of the liver from a patient with immediate preparation and culture of the hepatocytes *in vitro*. The cells are transduced with a retroviral vector as described below that is designed to produce for example Factor VIII for the treatment of hemophilia A. The cells are then transplanted back into the portal circulation where they will take residence in the liver indefinitely. Serum Factor VIII is monitored by RIA or ELISA with a specific antibody before and after treatment. The blood clotting parameters are monitored to establish efficacy.

A retroviral vector constructed similar to ones shown in figure 11 contains the human Factor VIII cDNA under the transcriptional control of the albumin promoter-enhancer (Pinkert, C.A., et al., 1987, Gene and Devel. 1:268-76) or other suitable promoter. The construct is transfected into an amphotropic packaging cell line and high titer colonies are isolated as described above. Amphotropic retroviral vectors infect hepatocytes with a relatively high transduction efficiency. The transduction efficiency are monitored by DNA blots from samples obtained from infected hepatocytes. Additionally, the Factor VIII production is measured *in vitro* by measuring the amount of this specific protein secreted into the media. The albumin promoter is used in this present construction because it represents a strong liver-specific promoter however, other promoters may be substituted as necessary.

The production of Factor VIII for gene therapy in the liver can also be carried out by other methods. These methods involve other viral or non viral vectors for *in vitro* as well as direct *in vivo* delivery to hepatocytes. These include adenovirus, adeno-associated virus, vaccinia virus, herpes virus, liposome-DNA complexes, protein cDNA complexes, and chemical-DNA complexes. Finally, the ability to express the Factor VIII gene to treat hemophilia is not limited to the liver. With the judicious choice of tissue specific promoters, the gene can be targeted to a variety of organs and tissues to achieve Factor VIII production and secretion into blood to achieve the medical benefits.

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The transformed hepatocytes, retroviral vectors, DNA vectors, along with the methods, procedures, treatments, molecules and specific compounds described herein are presently representative of preferred embodiments, are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

Claims

1. A method for treating diabetes mellitus comprising the step of:

introducing into an animal a nucleic acid vector encoding for expression of at least a functional portion of a proinsulin or insulin molecule; and

wherein said nucleic acid vector is capable of transforming a cell *in vivo* and expressing at least said functional portion of said proinsulin or insulin molecule in said transformed cell.

2. A method for treating diabetes mellitus comprising the steps of:

a) *in vitro* formation of a transformed cell by introducing a nucleic acid vector into a cell, wherein said vector contains a nucleic acid fragment encoding at least a functional portion of said sequence for an proinsulin or insulin molecule; wherein said nucleic acid vector is capable of expressing at least said functional portion of said proinsulin or insulin molecule in said transformed cell; and

b) administering said transformed cell to an animal.

3. The method according to claims 1 or 2, wherein said transformed cell is any cell not normally capable of producing insulin.

4. The method according to claims 1 or 2, wherein said transformed cell is a hepatocyte.

5. The method according to claims 1 or 2, wherein said transformed cell produces a constitutive level of proinsulin or insulin.

6. The method of according to claims 1 or 2, wherein said transformed cell is capable of regulated production

of proinsulin or insulin in response to a fluctuation in blood levels of insulin.

7. The method of according to claims 1 or 2, wherein said transformed cell is capable of regulated production of proinsulin or insulin in response to a fluctuation in blood levels of glucose.

8. The method of according to claims 1 or 2, wherein said nucleic acid vector is selected from the group consisting of a retroviral vector, an adenoviral vector an adenovirus-associated vector a herpes virus vector or a vaccinia virus vector.

9. The method of according to claims 1 or 2, wherein said nucleic acid vector is a formulated nucleic acid vector.

10. The method of according to claims 1 or 2, wherein said nucleic acid vector is selected from the group consisting of LX/rINS and LNCX.

11. The method of claim 1 or 2 further comprising the step of transforming said cell with a second nucleic acid vector containing a nucleic acid fragment encoding at least a functional portion of the A or B chain of said insulin molecule and wherein said nucleic acid vector of claim 1 or 2 contains at least a functional portion of the A chain of said insulin molecule when said second nucleic acid vector contains said B chain of said insulin molecule or wherein said nucleic acid vector of claim 1 or 2 contains at least a functional portion of said B chain when said second nucleic acid vector contains said A chain of said insulin molecule.

12. A method for modifying a peptidase cleavage consensus sequence in a protein so that a non-native

peptidase will cleave at said consensus sequence comprising the steps of:

a) identifying a peptidase cleavage consensus sequence of said non-native peptidase;

b) mutagenizing said peptidase cleavage consensus sequence of said protein so that it is identical to said peptidase cleavage consensus sequence of said non-native peptidase.

13. The method according to claim 12 wherein said peptidase consensus sequence comprises the proinsulin B chain/C chain peptidase consensus sequence.

14. The method according to claim 12 wherein said peptidase consensus sequence comprises the proinsulin A chain/C chain peptidase consensus sequence and said consensus sequence is modified to allow cleavage by a hepatic endopeptidase.

15. The method of claim 14 wherein said peptidase cleavage consensus sequence after modification is Arg-X-Lys-Arg or Arg-X-Arg-Arg, wherein X can be any amino acid.

16. The method according to claims 1 or 2 wherein the proinsulin sequence has been modified, said modification comprises altering the bases at the A chain/C chain peptidase consensus sequence to conform to the cleavage consensus sequence of a non-native endopeptidase.

17. The method of claim 16, wherein the cleavage consensus sequence after modification is Arg-X-Lys-Arg or Arg-X-Arg-Arg, wherein X can be any amino acid.

18. The method according to claims 1 or 2 wherein the proinsulin sequence has been modified, said modification comprises altering the bases at the B chain/C

chain peptidase consensus sequence to conform to the cleavage consensus sequence of a non-native endopeptidase.

19. A method for treating Parkinson's disease comprising the step of:

introducing into an animal a nucleic acid vector encoding for expression of at least a functional portion of tyrosine hydroxylase; and

wherein said vector is capable of transforming a cell *in vivo* and capable of expressing at least said functional portion of tyrosine hydroxylase in said transformed cell.

20. A method for treating Parkinson's disease comprising the steps of:

a) *in vitro* formation of a transformed cell by introducing a nucleic acid vector into a cell, wherein said vector contains a nucleic acid fragment encoding at least the functional portion of the sequence for tyrosine hydroxylase; wherein said cassette is capable of expressing tyrosine hydroxylase in the transformed cell; and

b) administering said transformed cell to an animal.

21. The method according to claims 19 or 20, wherein said transformed cell is a hepatocyte.

22. The method according to claims 19 or 20, wherein said transformed cell produces a constitutive level of at least a functional portion of tyrosine hydroxylase.

23. The method according to claims 19 or 20, wherein said transformed cell is capable of regulated production of tyrosine hydroxylase in response to regulatory molecules.

24. A method for treating cardiovascular disease comprising the step of:

introducing into an animal a nucleic acid vector encoding for expression of at least a functional portion of a protein selected from the group consisting of apolipoprotein A1, lipoprotein lipase, apolipoprotein E, cholesterol-7α-hydroxylase, and combinations thereof; and

wherein said nucleic acid vector is capable of transforming a cell *in vivo* and expressing at least said functional portion of said protein in said transformed cell.

25. A method for treating cardiovascular disease comprising the steps of:

a) *in vitro* formation of a transformed cell by introducing of a nucleic acid vector into a cell, wherein said vector contains a nucleic acid fragment encoding for expression of at least a functional portion of a protein selected from the group consisting of apolipoprotein A1, lipoprotein lipase, apolipoprotein E, cholesterol-7α-hydroxylase, and combinations thereof; wherein said vector is capable of expressing at least a functional portion of a protein selected from the group consisting of apolipoprotein A1, lipoprotein lipase, apolipoprotein E, cholesterol-7α-hydroxylase, and combinations thereof in the transformed cell; and

b) administering said transformed cell to an animal.

26. The method according to claims 24 or 25, wherein said transformed cell is a hepatocyte.

27. The method according to claims 24 or 25, wherein said transformed cell produces a constitutive level of at least a functional portion of said protein selected from the group consisting of apolipoprotein A1, lipoprotein lipase, apolipoprotein E, cholesterol-7α-hydroxylase, and combinations thereof.

28. The method according to claims 24 or 25, wherein said transformed cell is capable of regulated production of the protein selected from the group consisting of apolipoprotein A1, lipoprotein lipase, apolipoprotein E, cholesterol-7α-hydroxylase, and combinations thereof in response to regulatory molecules.

29. A method for treating hypercholesterolemia comprising the step of:

introducing into an animal a nucleic acid vector encoding for expression of at least a functional portion of an LDL receptor; and

wherein said nucleic acid vector is capable of transforming a cell *in vivo* and expressing at least said functional portion of said LDL receptor in said transformed cell.

30. A method for treating hypercholesterolemia comprising the steps of:

a) *in vitro* formation of a transformed cell by introducing of a nucleic acid vector into a cell, wherein said vector contains a nucleic acid fragment encoding for expression of at least a functional portion of an LDL receptor; wherein said vector is capable of expressing at least said functional portion of said LDL receptor in the transformed cell; and

b) administering said transformed cell to an animal.

31. The method according to claims 29 or 30, wherein said transformed cell is a hepatocyte.

32. The method according to claims 29 or 30, wherein said transformed cell produces a constitutive level of at least a functional portion of said LDL receptor.

33. The method according to claims 29 or 30, wherein said transformed cell is capable of regulated production of LDL receptor in response to regulatory molecules.

34. A method for treating hypertension comprising the step of:

introducing into an animal a nucleic acid vector encoding for expression of at least a functional portion of an ANF or γMSH molecule; and

wherein said nucleic acid vector is capable of transforming a cell *in vivo* and expressing at least said functional portion of said ANF or γMSH molecule in said transformed cell.

35. A method for treating hypertension comprising the steps of:

a) *in vitro* formation of a transformed cell by introducing of a nucleic acid vector into a cell, wherein said vector contains a nucleic acid fragment encoding for expression of at least a functional portion of an ANF or γMSH molecule; wherein said vector is capable of expressing at least said functional portion of said ANF molecule in the transformed cell; and

b) administering said transformed cell to an animal.

36. The method according to claims 34 or 35, wherein said transformed cell is a hepatocyte.

37. The method according to claims 34 or 35, wherein said transformed cell produces a constitutive level of at least a functional portion of said ANF or γMSH molecule.

38. The method according to claims 34 or 35, wherein said transformed cell is capable of regulated production of an ANF or γMSH molecule in response to regulatory molecules.

39. A method for treating anemia comprising the step of:

introducing into an animal a nucleic acid vector encoding for expression of at least a functional portion of an EPO molecule; and

wherein said nucleic acid vector is capable of transforming a cell *in vivo* and expressing at least said functional portion of said EPO molecule in said transformed cell.

40. A method for treating anemia comprising the steps of:

a) *in vitro* formation of a transformed cell by introducing of a nucleic acid vector into a cell, wherein said vector contains a nucleic acid fragment encoding for expression of at least a functional portion of an EPO molecule; wherein said vector is capable of expressing at least said functional portion of said EPO molecule in the transformed cell; and

b) administering said transformed cell to an animal.

41. The method according to claims 39 or 40, wherein said transformed cell is a hepatocyte.

42. The method according to claims 39 or 40, wherein said transformed cell produces a constitutive level of at least a functional portion of said EPO molecule.

43. The method according to claims 39 or 40, wherein said transformed cell is capable of regulated production of an EPO molecule in response to regulatory molecules.

44. A method for treating thrombosis comprising the step of:

introducing into an animal a nucleic acid vector encoding for expression of at least a functional portion of a protein selected from the group consisting of

thrombomodulin, protein C, protein S, and antithrombin-III, and combinations thereof; and

wherein said nucleic acid vector is capable of transforming a cell *in vivo* and expressing at least said functional portion of said protein selected from the group consisting of thrombomodulin, protein C, protein S, and antithrombin-III, and combinations thereof in said transformed cell.

45. A method for treating thrombosis comprising the steps of:

a) *in vitro* formation of a transformed cell by introducing of a nucleic acid vector into a cell, wherein said vector contains a nucleic acid fragment encoding for expression of at least a functional portion of a protein selected from the group consisting of thrombomodulin, protein C, protein S, and antithrombin-III, and combinations thereof; wherein said vector is capable of expressing said protein selected from the group consisting of thrombomodulin, protein C, protein S, and antithrombin-III, and combinations thereof in the transformed cell; and

b) administering said transformed cell to an animal.

46. The method according to claims 44 or 45, wherein said transformed cell is a hepatocyte.

47. The method according to claims 44 or 45, wherein said transformed cell produces a constitutive level of at least a functional portion of said proteins selected from the group consisting of thrombomodulin, protein C, protein S, and antithrombin-III, and combinations thereof.

48. The method according to claims 44 or 45, wherein said transformed cell is capable of regulated production of the protein selected from the group consisting of thrombomodulin, protein C, protein S, and

antithrombin-III, and combinations thereof in response to regulatory molecules.

49. A method for supplying growth hormone to an animal comprising the step of:

introducing into an animal a nucleic acid vector encoding for expression of at least a functional portion of a growth hormone molecule; and

wherein said nucleic acid vector is capable of transforming a cell *in vivo* and expressing at least said functional portion of said growth hormone molecule in said transformed cell.

50. A method for supplying growth hormone to an animal comprising the steps of:

a) *in vitro* formation of a transformed cell by introducing of a nucleic acid vector into a cell, wherein said vector contains a nucleic acid fragment encoding for expression of at least a functional portion of a growth hormone molecule; wherein said vector is capable of expressing at least said functional portion of said growth hormone molecule in the transformed cell; and

b) administering said transformed cell to an animal.

51. The method according to claims 49 or 50, wherein said transformed cell is a hepatocyte.

52. The method according to claims 49 or 50, wherein said transformed cell produces a constitutive level of at least a functional portion of said growth hormone molecule.

53. The method according to claims 49 or 50, wherein said transformed cell is capable of regulated production of a growth hormone molecule in response to regulatory molecules.

54. A method for treating metabolic disorders comprising the step of:

introducing into an animal a nucleic acid vector encoding for expression of at least a functional portion of a protein selected from the group consisting of PAH, methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase, and combinations thereof; and

wherein said nucleic acid vector is capable of transforming a cell *in vivo* and expressing at least said functional portion of said protein selected from the group consisting of PAH, methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase, and combinations thereof in said transformed cell.

55. A method for treating metabolic disorders comprising the steps of:

a) *in vitro* formation of a transformed cell by introducing of a nucleic acid vector into a cell, wherein said vector contains a nucleic acid fragment encoding for expression of at least a functional portion of a protein selected from the group consisting of PAH, methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase, and combinations thereof; wherein said vector is capable of expressing said protein selected from the group consisting of PAH, methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase, and combinations thereof in the transformed cell; and

b) administering said transformed cell to an animal.

56. The method according to claims 54 or 55, wherein said transformed cell is a hepatocyte.

57. The method according to claims 54 or 55, wherein said transformed cell produces a constitutive level of at least a functional portion of said proteins selected from the group consisting of PAH, methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase, and combinations thereof.

58. The method according to claims 54 or 55, wherein said transformed cell is capable of regulated production of the protein selected from the group consisting of PAH, methylmalonyl CoA mutase, proprionyl CoA carboxylase, ornithine transcarbamylase, carbamylphosphate synthetase and arginase-arginosuccinate lyase, and combinations thereof in response to regulatory molecules.

59. A method for treating serum protein disorders comprising the step of:

introducing into an animal a nucleic acid vector encoding for expression of at least a functional portion of a Factor VIII molecule; and

wherein said nucleic acid vector is capable of transforming a cell *in vivo* and expressing at least said functional portion of said Factor VIII molecule in said transformed cell.

60. A method for treating serum protein disorders comprising the steps of:

a) *in vitro* formation of a transformed cell by introducing of a nucleic acid vector into a cell, wherein said vector contains a nucleic acid fragment encoding for expression of at least a functional portion of a Factor VIII molecule; wherein said vector is capable of expressing at least said functional portion of said Factor VIII molecule in the transformed cell; and

b) administering said transformed cell to an animal.

61. The method according to claims 59 or 60, wherein said transformed cell is a hepatocyte.

62. The method according to claims 59 or 60, wherein said transformed cell produces a constitutive level of at least a functional portion of said Factor VIII molecule.

63. The method according to claims 59 or 60, wherein said transformed cell is capable of regulated production of a Factor VIII molecule in response to regulatory molecules.

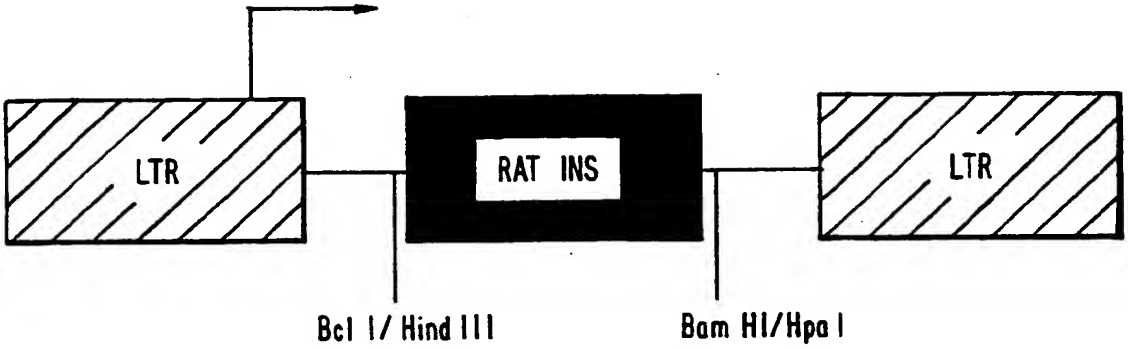

LTR
RAT INS
LTR
Bcl I/ Hind III
Bam HI/Hpa I

FIG. 1a.

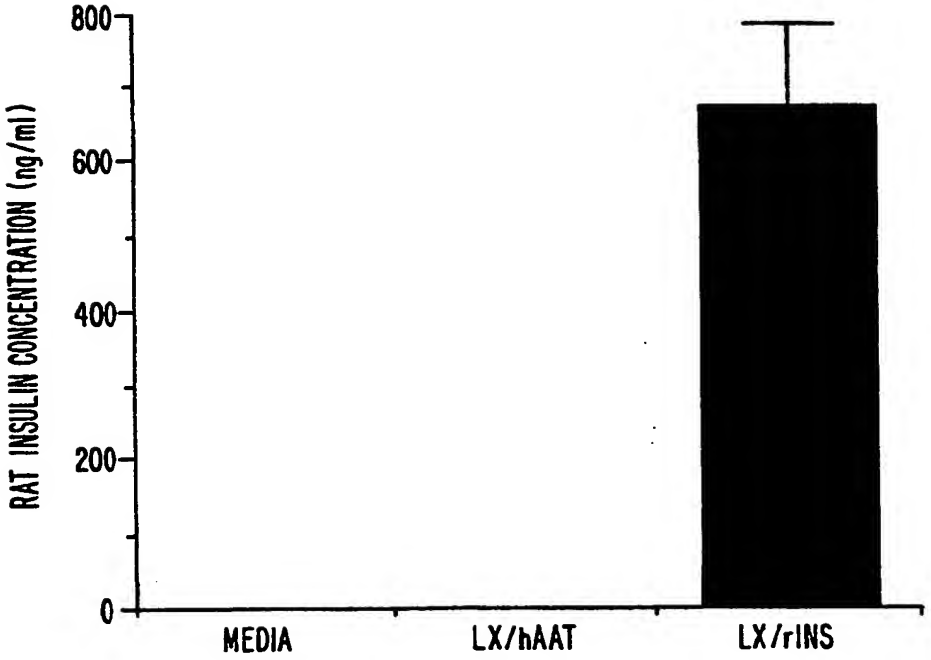

800
600
400
200
0
RAT INSULIN CONCENTRATION (ng/ml)
MEDIA
LX/hAAT
LX/rINS

FIG. 1b.

FIG. 2a.

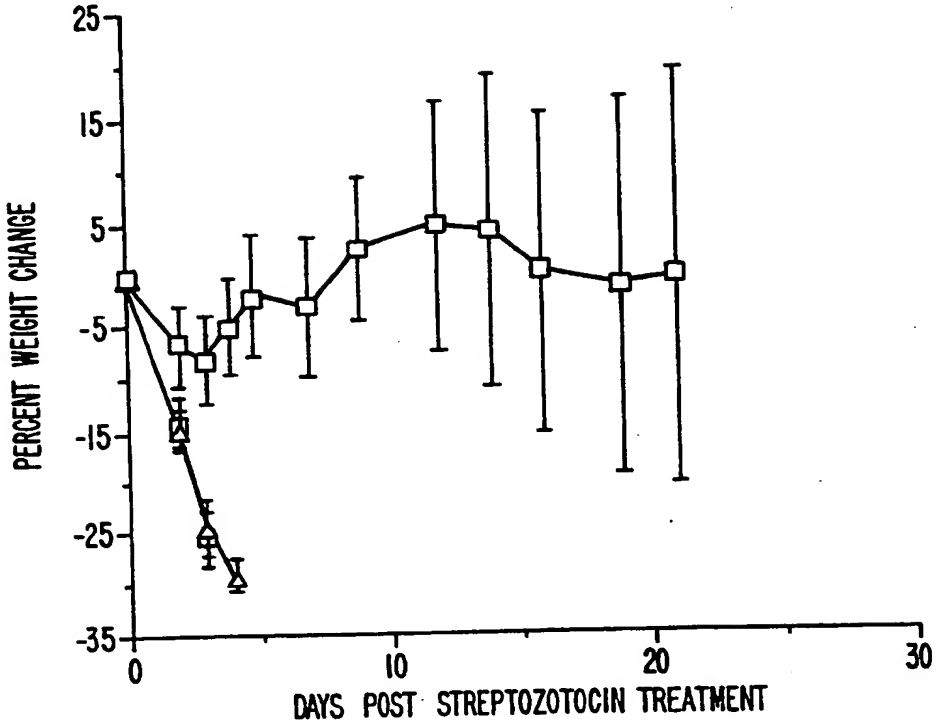

25
15
5
-5
-15
-25
-35
PERCENT WEIGHT CHANGE
0
10
20
30
DAYS POST STREPTOZOTOCIN TREATMENT

FIG. 2b.

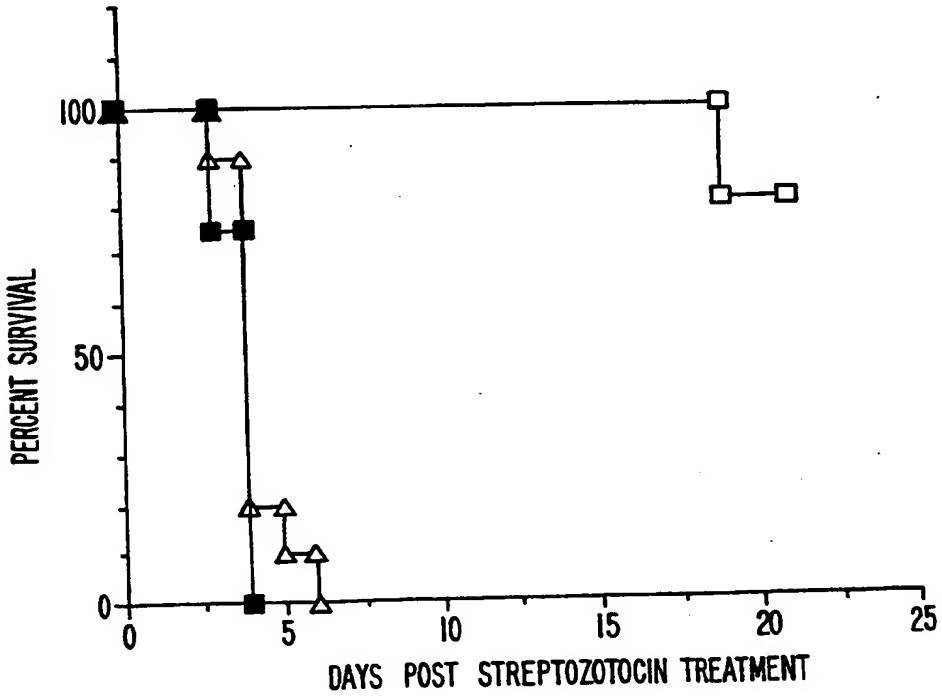

100
50
0
PERCENT SURVIVAL
0
5
10
15
20
25
DAYS POST STREPTOZOTOCIN TREATMENT

FIG. 3a.

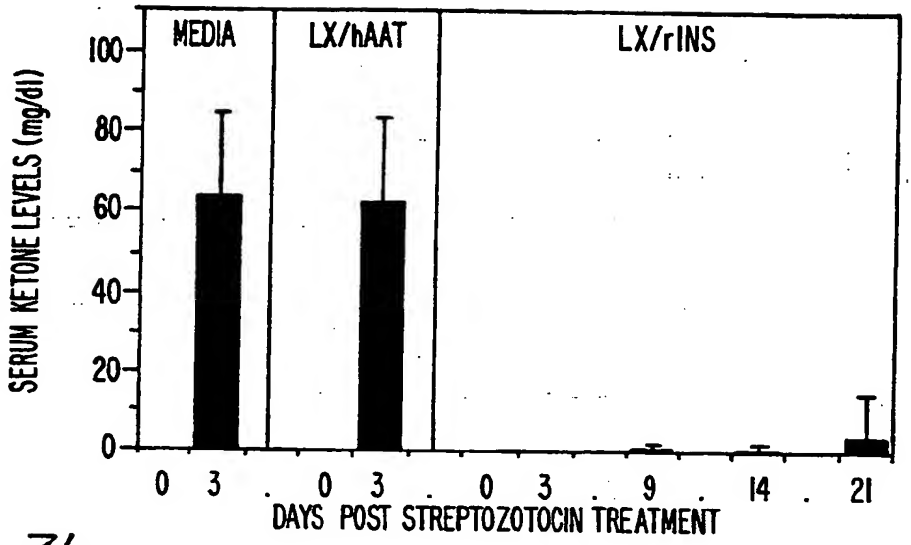


FIG. 3b.

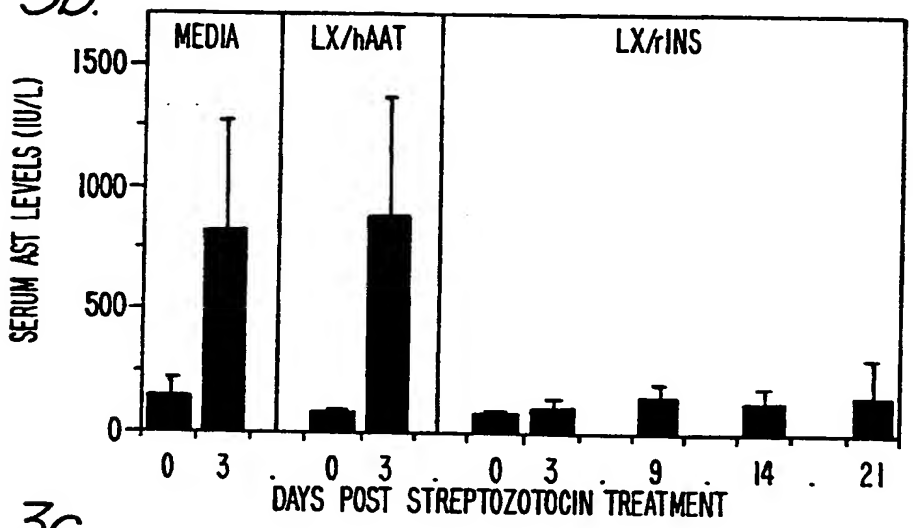


FIG. 3c.

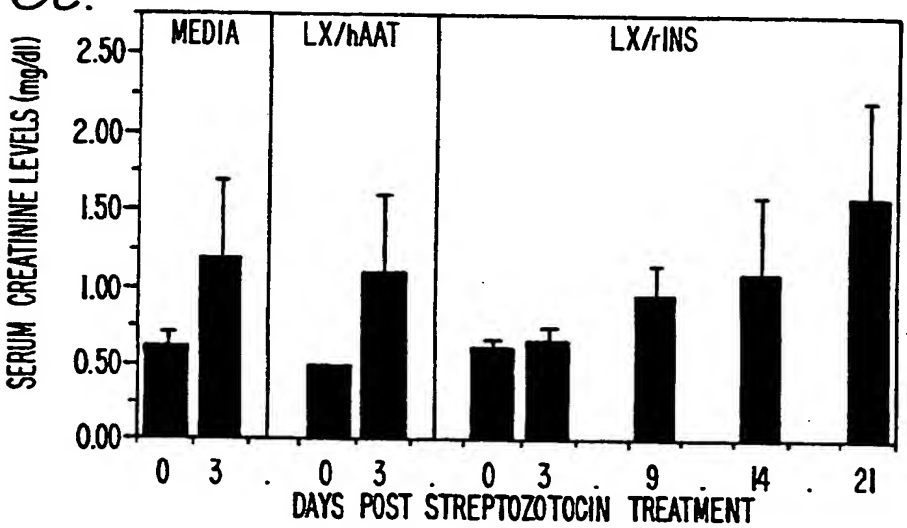


FIG. 4a.

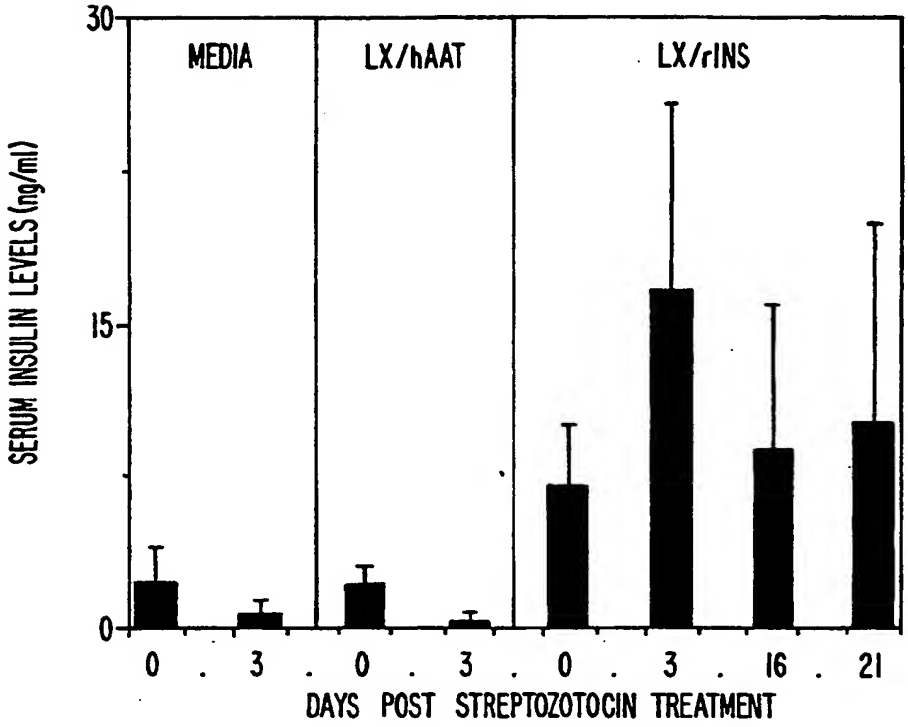


FIG. 4b.

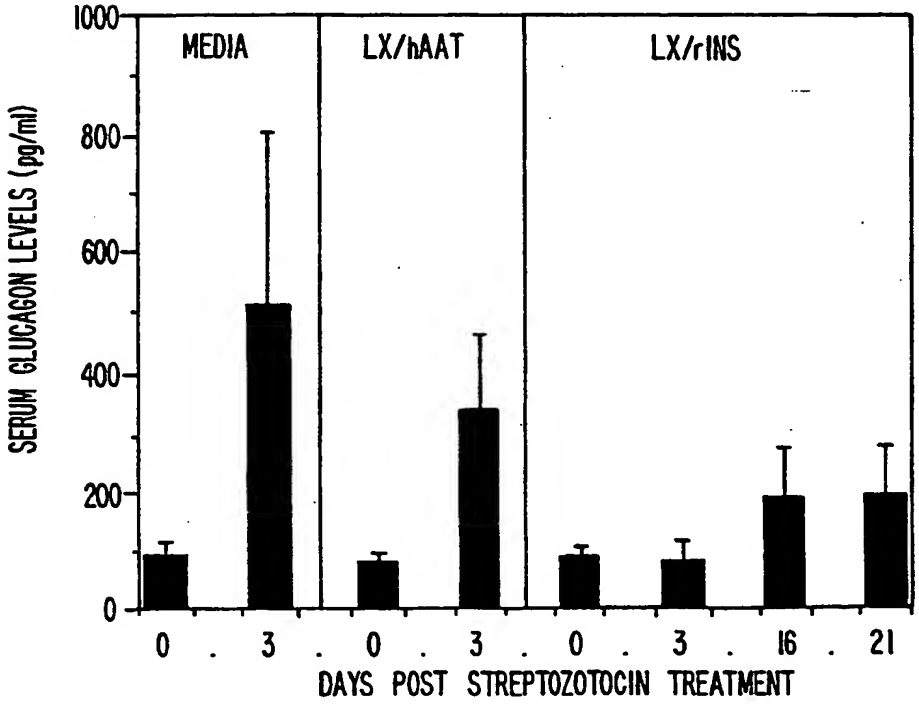


FIG. 5a.

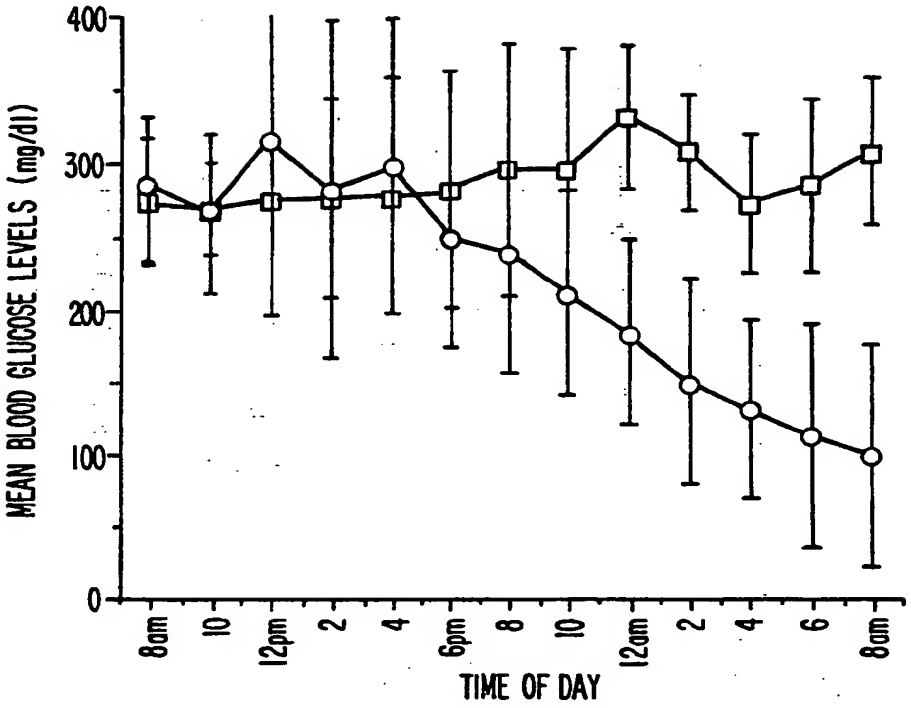
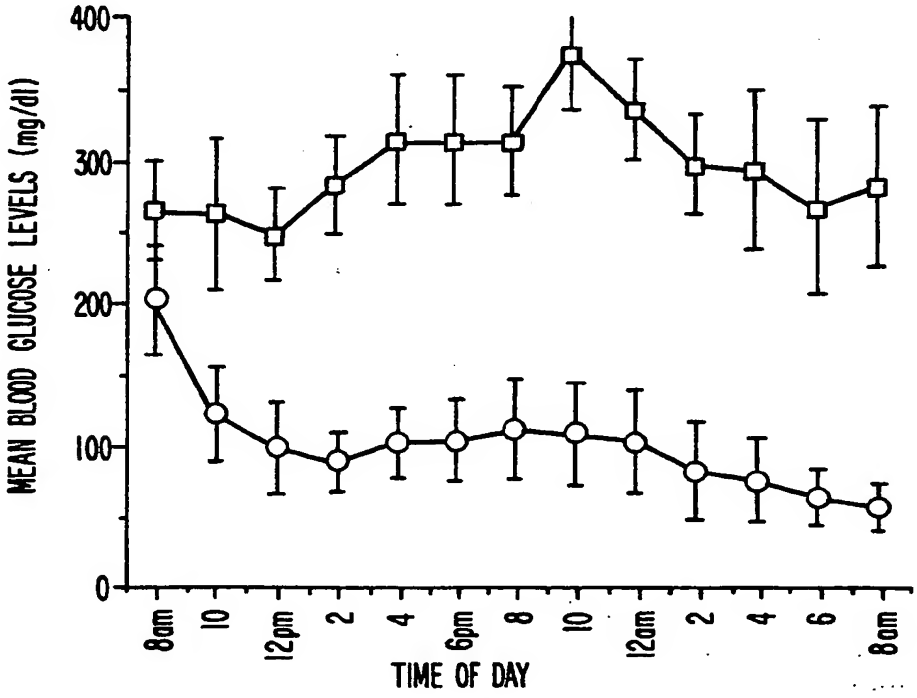

MEAN BLOOD GLUCOSE LEVELS (mg/dl)
400
300
200
100
0
8am
10
12pm
2
4
6pm
8
10
12am
2
4
6
8am
TIME OF DAY

FIG. 5b.


MEAN BLOOD GLUCOSE LEVELS (mg/dl)
400
300
200
100
0
8am
10
12pm
2
4
6pm
8
10
12am
2
4
6
8am
TIME OF DAY

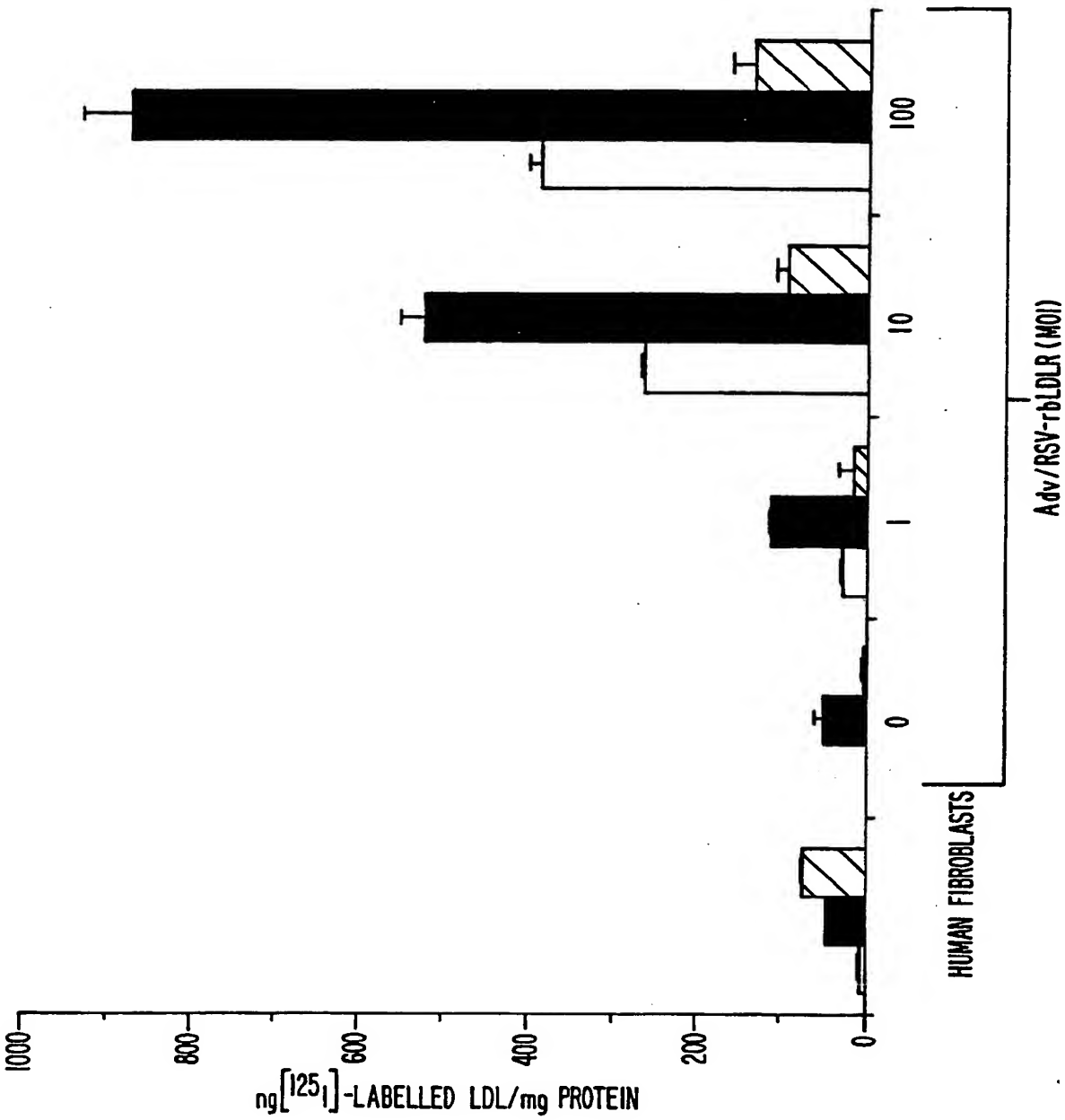


FIG. 6.

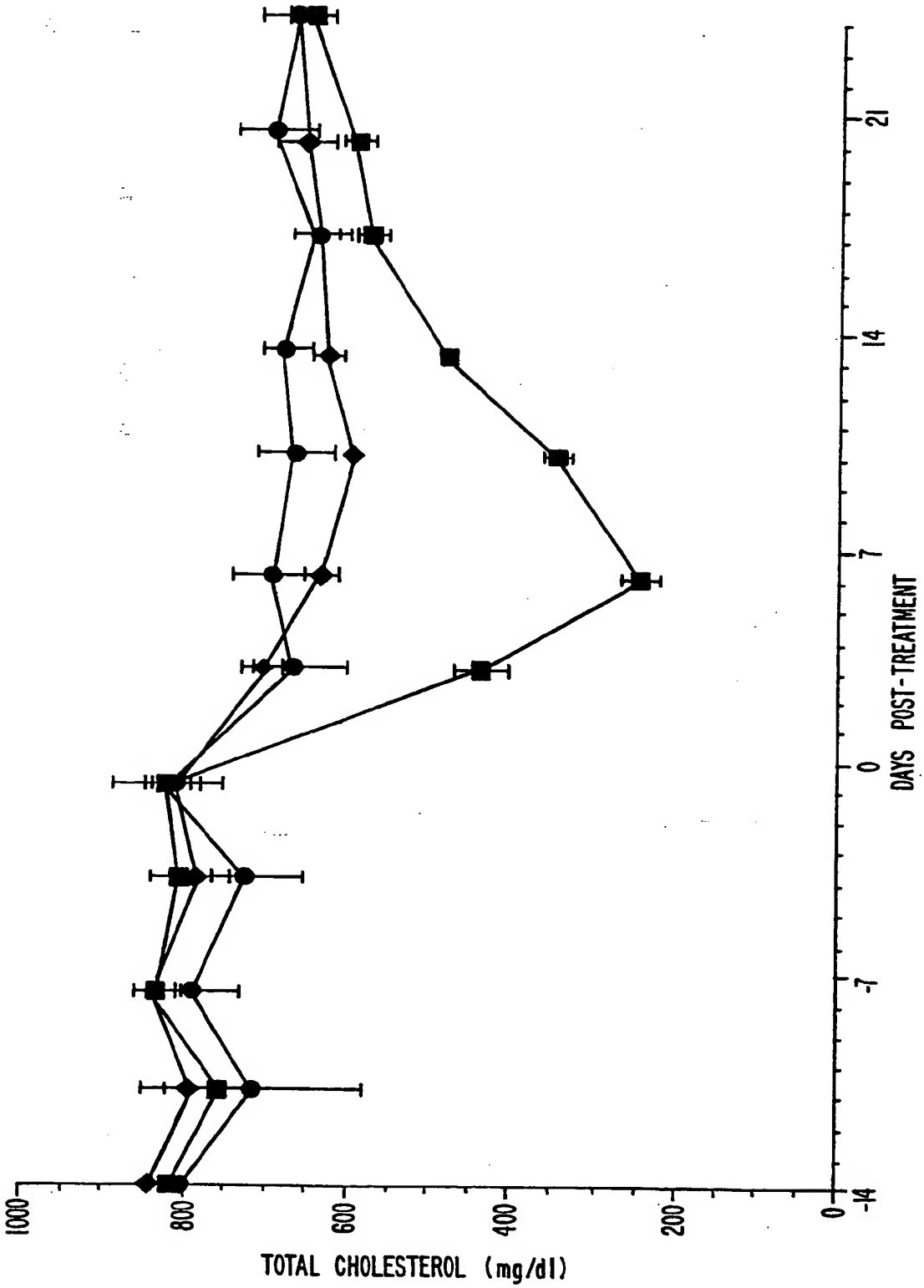
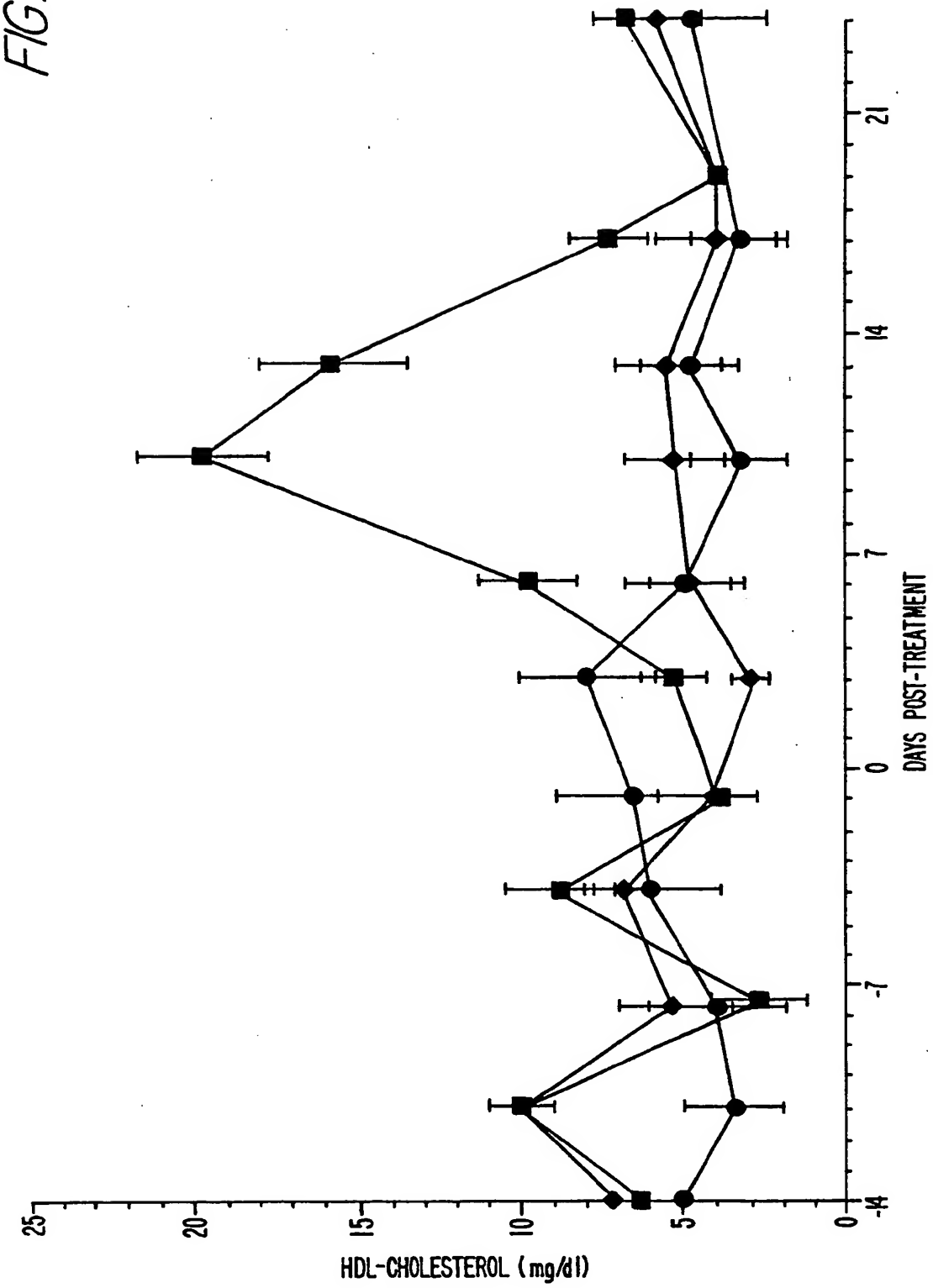

1000
800
600
400
200
0
TOTAL CHOLESTEROL (mg/dl)
-14
-7
0
7
14
21
DAYS POST-TREATMENT

FIG. 7.

FIG. 8.



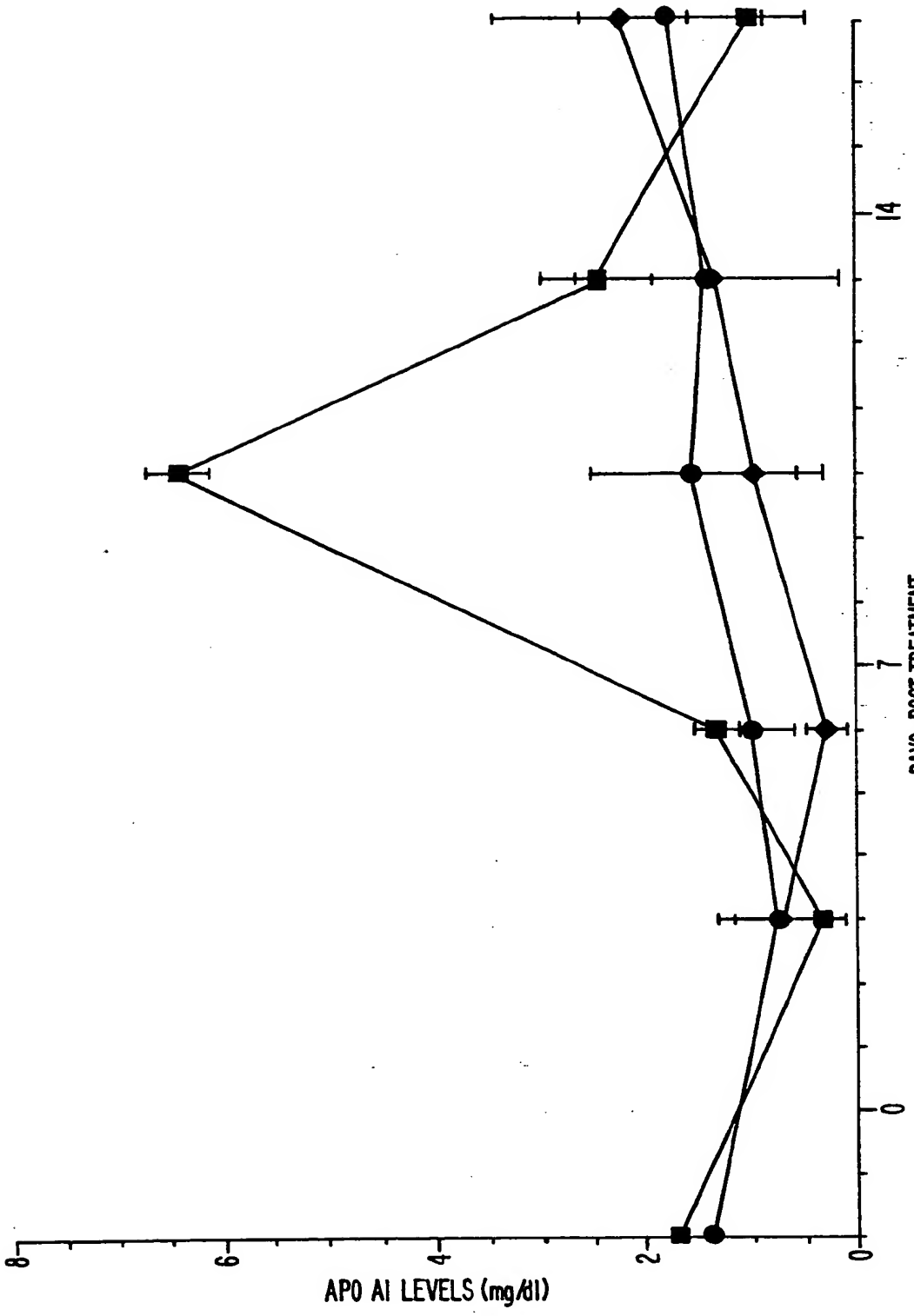


FIG. 9.

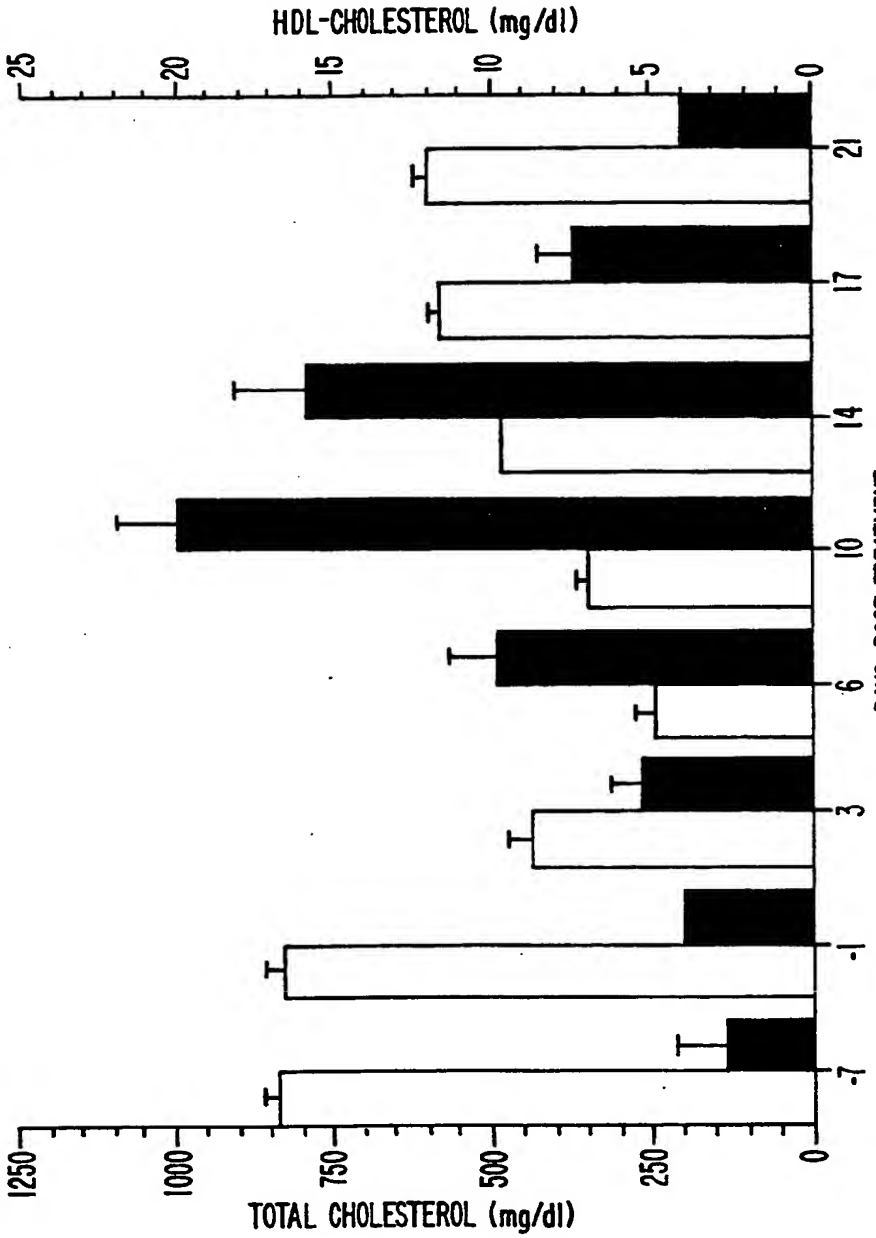


FIG. 10.

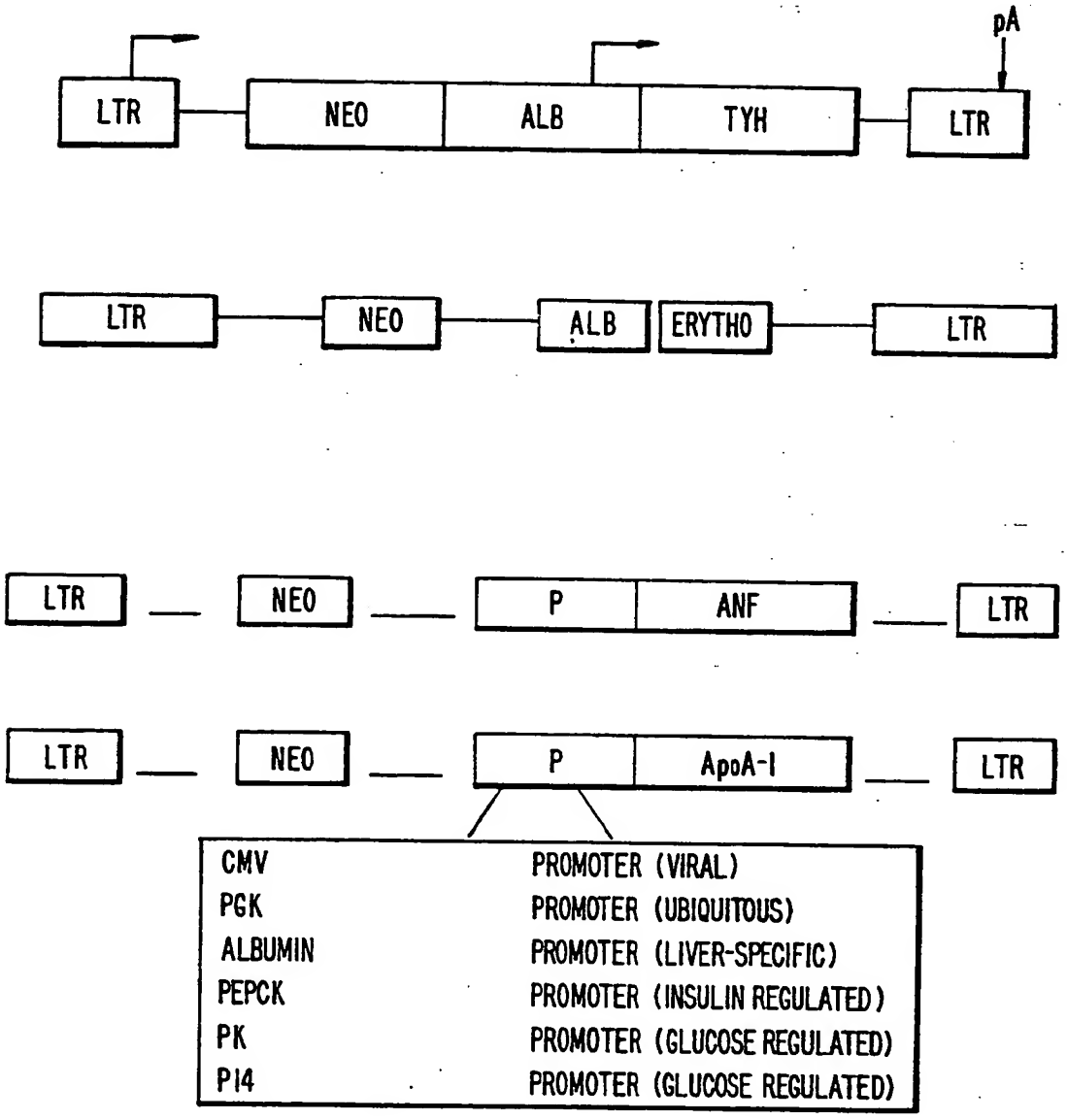

pA
LTR
NEO
ALB
TYH
LTR
LTR
NEO
ALB
ERYTHO
LTR
LTR
NEO
P
ANF
LTR
LTR
NEO
P
ApoA-I
LTR
CMV PROMOTER (VIRAL)
PGK PROMOTER (UBIQUITOUS)
ALBUMIN PROMOTER (LIVER-SPECIFIC)
PEPCK PROMOTER (INSULIN REGULATED)
PK PROMOTER (GLUCOSE REGULATED)
P14 PROMOTER (GLUCOSE REGULATED)

FIG. 11.